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SHORT COMMUNICATIONS

Acta Agronomica Hungarica, 58(1), pp. 1–10 (2010)
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ANALYSIS OF LATERAL ROOT GROWTH IN *ARABIDOPSIS* IN RESPONSE TO PHYSIOLOGICALLY ACTIVE AUXIN ANALOGUES

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The aim of this work was to investigate the formation and development of lateral roots in model trials on *Arabidopsis thaliana* L. Heynh wild type (Col-0), the *alf4-1* mutant and its allele by applying the physiologically active auxin analogues IBA, IAA, TA-12 and TA-14.

Differences were observed between the *alf4-1* mutant and its allele phenotype in the formation of lateral roots. The application of auxin analogues was unable to restore the formation of lateral roots in the *alf4-1* mutant. In some cases, under the impact of IBA (1 μM), a cluster of xylem cells was activated in the pericycle of the primary roots and lateral root primordia were formed. The auxin analogues induced the growth of primary roots in the *alf4-1* allele and the formation and growth of lateral roots. The impact of IBA (1 μM), TA-12 (1 mM) and IAA (1 μM) was particularly evident. The intense formation of lateral roots under the impact of IBA and TA-12 could be related with the ability of these compounds to intensify mitotic activity in the apical meristem cells of the lateral roots. New data were obtained, showing that IBA and other physiologically active auxin analogues can modify the root system architecture of the test-plant *Arabidopsis*.

Key words: *Arabidopsis*, *alf4-1* mutant, auxin analogues, lateral root

Abbreviations: IAA – 3-indolylacetic acid, IBA – 3-indolylbutyric acid, TA-12 – calcium 4-(2-chlorethoxycarbonylmethyl)-1-naphthalenesulphonate, TA-14 – ω-trialkyl-ammonioalkyl ester of 1-naphthylethanoic acid, LR – lateral root

Introduction

Lateral root (LR) formation is regulated by both developmental programmes and environmental conditions. LRs develop from founder cells in the pericycle, the outermost layer of the vascular cylinder of the root. The process of LR formation in *Arabidopsis* can be divided into several stages: stimulation and dedifferentiation of pericycle cells (Dubrovsky et al., 2000); ordered cell divisions and cell differentiation to generate a highly organized lateral root primordium (Malamy and Benfey, 1997); emergence via cell

expansion (Benkova et al., 2003); activation of the LR primordium meristem to allow continued growth of the organized LR (Casimiro et al., 2001).

Although the mechanism controlling LR formation is not fully understood, it is obvious that auxin plays a dominant role in these processes (Casimiro et al., 2001; Benkova et al., 2003). The role of physiologically active auxin analogues, especially 3-indolylbutyric acid (IBA), in LR induction and development, and in processes involved in the formation of root system architecture is also little known. It is becoming clear that besides the classical auxin IAA, other auxin-like substances, such as IBA, exist in plants (Ludwig-Müller, 2000; 2007). At lower concentrations IBA was also more effective than IAA in promoting lateral root growth in *Arabidopsis* (Zolman et al., 2000). Some data indicate that synthetic auxins such as naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and the original physiological auxin analogue TA-12 stimulate lateral or adventitious root formation in horticultural and agricultural plants (Novickienė and Darginavičienė, 2001; Miliuvienė et al., 2006; Gavelienė et al., 2007).

In recent years it has become possible to study LR induction, development and root system architecture formation using *Arabidopsis* mutants. Most LR mutants are affected in a specific part of the auxin pathway and their phenotypes can usually be rescued or mimicked through auxin application (Bao et al., 2007). It is known that *alf4-1* mutation prevents the initiation of lateral roots. *Alf4-1* pericycle cells are impeded in completing mitosis, thus preventing lateral root development. *ALF4* acts downstream and possibly independent of the auxin signal (Celenza et al., 1995; DiDonato et al., 2004).

The aim of the present work was to study the formation and development of LRs in model trials on *Arabidopsis thaliana* L. Heynh wild type (Col-0), the *alf4-1* mutant and its allele, to investigate the possibility of restoring LR induction by applying for the first time the physiologically active auxin analogues IBA, TA-12 and TA-14, and to compare their effects with that produced by 3-indolylacetic acid (IAA)

Materials and methods

The experiments were carried out on *Arabidopsis thaliana* wild type (Col-0), on the *alf4-1* mutant, kindly provided by Prof. John Celenza (Department of Biology, Boston University), and on the allele of this mutant, which was the kind gift of the Nottingham Arabidopsis Stock Centre (NASC).

3-indolylbutyric acid (IBA) and two other auxin analogues, TA-12, calcium 4-(2-chloroethoxycarbonylmethyl)-1-naphthalenesulphonate (Merkys et al., 2006) and TA-14, the ω-trialkylammonioalkyl ester of 1-naphthylethanoic acid, were used for the first time to regulate the lateral root formation of *Arabidopsis* (Novickienė and Gavelienė, 2000).

To select the optimal concentrations of the physiological auxin analogues tested, trials on *Arabidopsis* wild type (Col-0) LR formation were carried out by germinating the seeds in aqueous solutions of the compounds at concentrations of 1 mM, 5 μM, 1 μM and 0.1 μM for 24 h, after which the seeds were transferred to Murashige-Skoog (MS) culture medium (Murashige and Skoog, 1962) at a constant temperature (22°C) under a 16:8 h light:dark cycle. After ten days the LR formation was measured. The morphometric results showed that the optimal concentrations were 1 μM for IAA, 1 μM for IBA, 1 mM for TA-12 and 5 μM for TA-14 (Fig. 1).

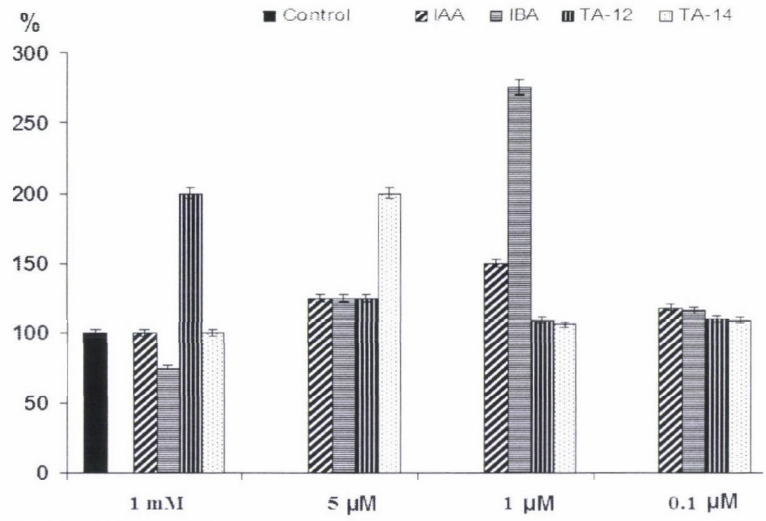

Fig. 1. Effect of auxin analogues on lateral root formation in *Arabidopsis* wild type

The lateral root development of *Arabidopsis* wild type (Col-0), the *alf4-1* mutant and its allele was then studied *in vitro* and the possibility of restoring LR induction by applying the auxin analogues IBA, IAA, TA-12 and TA-14 was investigated.

Arabidopsis seeds were surface-sterilized for 1 min in 75% ethanol, followed by 0.5% hypochlorite for 1 min and five washes with distilled water before sowing. The seeds were sown under sterile conditions in modified MS supplemented with 100 mg l^{-1} myoinositol, 0.1 mg l^{-1} thiamine HCl and 3% sucrose. The auxin analogues were added to the culture medium before sterilization at the optimal concentrations indicated above. Morphometric analysis of the primary and lateral roots was carried out after 10 or 15 days. For each variant 30 seedlings were analysed in 2 replications.

Cytological analysis of LR apical meristem cells

To estimate the mitotic activity of the lateral root apical meristem cells, the roots were fixed in Carnoy-mixture (50% ethanol and glacial acetic acid 3:1) after 10 days of treatment with the test compounds. After 4 days of fixation, the mixture was washed off the roots. The apical meristem zone was excised and stained with acetocarmine at a temperature of 80–90°C, after which the cell walls were macerated with chlorhydrate (Paulauskas et al., 2003). The cell mitosis phases and divided cells were counted in temporary squash preparations under a light microscope (AY-26) using a digital video camera (Olympus DP-11) and the mitotic index (MI) was calculated. MI is the cell number in mitosis per 1000 cells of the analysed objects (‰), i.e. MI=(M/N)×1000, where M is the number of mitoses and N is the cell number. For each variant 20 lateral root apical meristems were analysed.

Anatomical and histological analysis of LR primordium formation

The primary roots were excised from 10 seedlings in the control variant and in the variant tested with IBA after 4 days of treatment. The prepared samples were fixed in formalin, acetic acid and ethanol (1:1:20), dehydrated in a graded ethanol series, embedded in paraffin and cut with a rotary microtome into 10–15 μm sections (Kublickienė, 1978). Longitudinal deparaffined sections were stained with Schiff's reagent and analysed under a light microscope equipped with a digital video camera (Olympus, DP-11). The images were analysed using the Sigma Scan Pro (Jandel Scientific Software) programme.

Statistical analysis

The data were analysed using the descriptive statistics of the Microsoft Excel statistical program. The differences between the test variant and the control were significant at $P \leq 0.05$ (Songailienė and Ženauskas, 1985).

Results

The response patterns of primary and LR growth to auxin analogues were different in the wild type, the *alf4-1* mutant and its allele. The primary root length of the *alf4-1* allele was 35% longer after treatment with IBA and TA-12 than in the control. At the same time, IBA stimulated primary root growth by 28 and 16% in the *alf4-1* mutant and the wild type, respectively (Table 1).

The results showed differences between the *Arabidopsis alf4-1* mutant and its allele phenotype in LR formation. The application of physiological auxin analogues was unable to restore the induction of LR in *alf4-1* mutant seedlings. However, the investigation showed that in some cases, under the impact of IBA (1 μM) or TA-12 (1 mM), a few LR primordia were formed. It was also established that auxin analogues induced the formation and growth of LRs in the *alf4-1* allele, though the response was less pronounced than in the wild type (Table 1).

Table 1
Effect of physiologically active auxin analogues on root formation and development in *Arabidopsis* wild type, the *alf4-1* mutant and its allele

Test variant	Primary root length		Lateral roots			
	cm	%	No.	%	Length	
					mm	%
Wild type (Col-0)						
Control	2.5±0.6	100	6±0.10	100	3.0±0.2	100
IAA 1 μM	2.8±0.8	112	8±0.08	139	3.7±0.1	123
IBA 1 μM	2.9±0.5	116	10±0.21	166	4.0±0.2	133
TA-12 1 mM	2.7±0.2	108	10±0.09	166	3.8±0.1	126
TA-14 5 μM	2.7±0.3	108	9±0.08	150	4.0±0.1	133
alf4-1 allele						
Control	2.3±0.9	100	3±0.2	100	1.5±0.09	100
IAA 1 μM	2.6±0.5	118	5±0.4	166	1.9±0.10	127
IBA 1 μM	3.1±1.0	135	6±0.4	200	2.2±0.13	147
TA-12 1 mM	3.1±0.8	135	6±0.5	200	2.5±0.10	167
TA-14 5 μM	3.0±0.7	130	5±0.3	133	2.1±0.10	140
alf4-1 mutant						
Control	1.8±1.3	100				
IAA 1 μM	2.1±1.0	117				
IBA 1 μM	2.3±0.9	128	A few lateral root primordia were observed			
TA-12 1 mM	2.1±1.1	117	A few lateral root primordia were observed			
TA-14 5 μM	2.2±1.2	122				

The data presented demonstrated that the most effective compounds for primary and lateral root formation in the allele were IBA and TA-12 (Figs. 2 and 3). Differences were observed in the phenotype of the *alf4-1* mutant and its allele with respect to LR formation (Fig. 3).

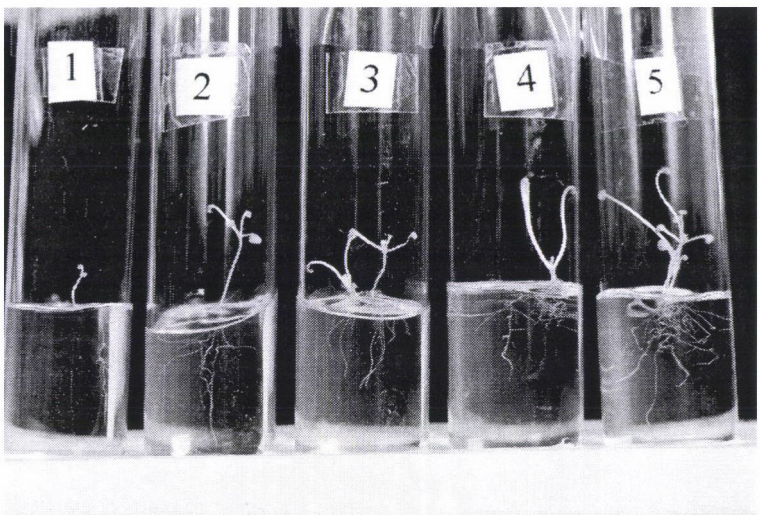

Fig. 2. Effect of auxin analogues on lateral root formation in the *alf4-1* allele: 1. Control, 2. IAA (1 μM), 3. TA-14 (5 μM), 4. TA-12 (1 mM), 5. IBA (1 μM)

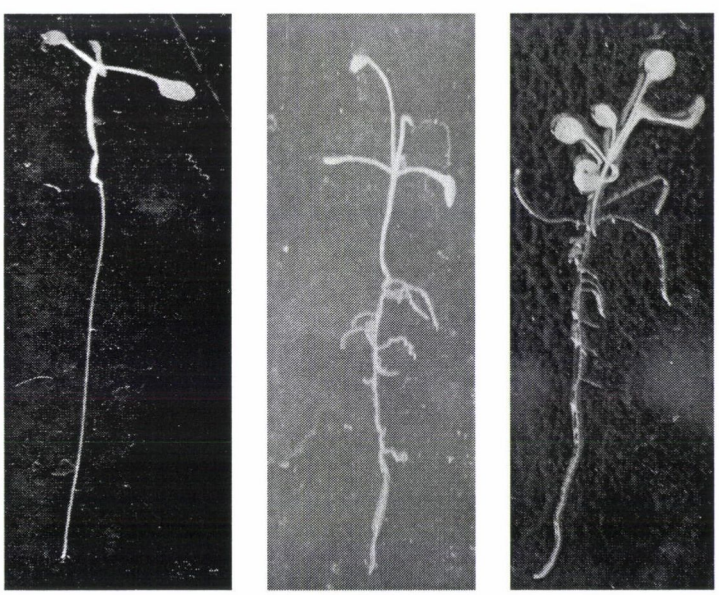

Fig. 3. Effect of IBA (1 μM) on primary and lateral root formation in the *Arabidopsis alf4-1* mutant (I), its allele (II) and the wild type (III) (after 10 days of growth) (×1.5)

The intense formation of LRs in response to IBA, TA-12, IAA and TA-14 could be related with the impact of these compounds on the mitotic index (MI) of the apical meristem cells of LRs in the allele. The highest MI was revealed in allele seedlings treated with IBA, TA-12 and IAA (53, 30 and 25%, respectively, in comparison with the control; Fig. 4). The ability of the most active compounds, IBA and TA-12, to intensify the division of LR meristem cells was then investigated. The cytological analysis of LR apical meristem cells in the allele showed that the tested compounds stimulated cell division. Cells were observed in prophase, anaphase and telophase after treatment with IBA and in prophase, metaphase and occasionally in telophase in the case of TA-12, whereas most cells in control seedlings were in prophase (Fig. 5).

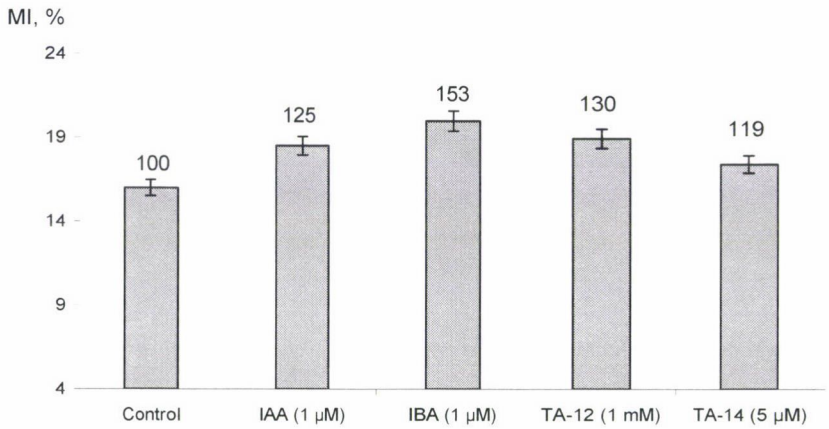

Fig. 4. Effect of auxin analogues on the mitotic index (MI) of lateral root meristem cells in the *alf4-1* allele (Control = 100 %)

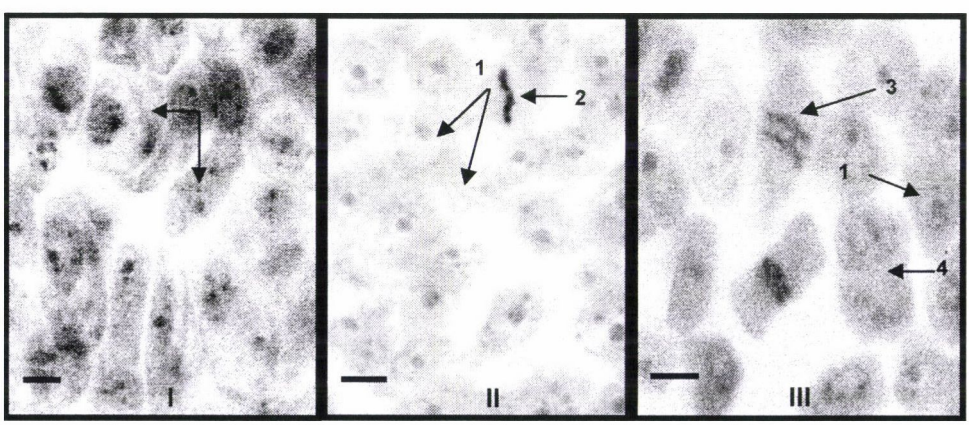

Fig. 5. Effect of TA-12 and IBA on the mitotic activity of the apical meristem cells in lateral roots of the *alf4-1* allele. I: Control; II: Ta-12 (1 mM); III: IBA (1 μM); 1. prophase; 2. metaphase; 3. anaphase; 4. telophase (Bar = 20 μm)

Earlier data showed that these auxin analogues could not restore LR induction and development in the *alf4-1* mutant, though LR primordia were formed in some cases under the effect of IBA and TA-14. Anatomical and histological methods were applied to clarity this situation. The anatomical analysis of longitudinal sections of *alf4-1* mutant primary roots revealed that the pericycle cells were unable to complete mitosis, thus preventing LR development. However, under the effect of IBA the pericycle cells adjacent to the xylem terminally differentiated and conglomerated, but LR did not form, though in some cases the primordium of LR was formed and penetrated through the epidermis (Fig. 6).

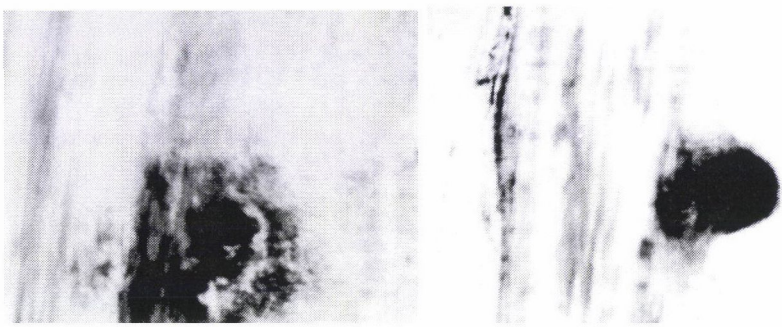

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Fig. 6. Effect of IBA (1 μM) on lateral root primordium formation in the *alf4-1* mutant (after 4 days of treatment): 1. conglomeration of cells in the primary root pericycle, 2. lateral root primordium

Discussion

It is known that the number of LR can be altered by the application of auxin or by the perturbation of internal auxin levels (Blakely et al., 1982; Kares et al., 1990). Auxin transported from the shoot is necessary for LR formation in young seedlings (Casimiro et al., 2001). Both literature references and our earlier data indicate that the auxin polar transport determines LR formation: the auxin transport inhibitors NPA (naphthylthalamic acid) or TIBA (2,3,5-triiodobenzoic acid) block auxin transport in the primary root, thus suppressing LR formation. However, the auxin analogues NAA (naphthaleneacetic acid) and TA-12 restore the auxin transport, resulting in LR formation (Casimiro et al., 2001; Miliuvienė et al., 2006; Gavelienė et al., 2007). Other authors stated that exogenous NAA and 2,4-D enhanced LR primordium initiation in *Arabidopsis*, but this process was more effective with NAA than with 2,4-D (Bao et al., 2007). However, in the LR initiation process only some of the pericycle cells respond to the auxin signal (Teale et al., 2005).

The number of LR is altered in mutants where the auxin metabolism is affected. The *alf4-1* mutation blocks the initiation of LR, thus greatly altering

root system architecture (Celenza et al., 1995; DiDonato et al., 2004). DiDonato et al. (2004) reported that the ALF4 protein is nuclear-localized and that *ALF4* expression and the subcellular location of ALF are not regulated by auxin. The present results showed differences between the *Arabidopsis alf4-1* mutant and its allele phenotype in the formation of lateral roots. The application of auxin analogues was not able to restore the formation of lateral roots in seedlings of the *Arabidopsis alf4-1* mutant. In some cases, under the impact of IBA (1 μM), the cluster of xylem cells was activated in the pericycle of primary roots, and the division of meristematic cells and the formation of lateral root primordia were observed. At the same time, auxin analogues induced the growth of primary roots in the *Arabidopsis alf4-1* allele, and the formation and growth of lateral roots. According to the data, the primary root length of the *alf4-1* mutant was 39% lower than that of the wild type and 27% less than in the allele. Under the impact of IBA the primary root length decreased by 28 % in the mutant, by 35% in the allele and by 16% in the wild type in comparison with the control. Less cell division was observed in the primary root tip of the *alf4-1* mutant, suggesting that *ALF4* may maintain mitotic competence in other root tissues besides the pericycle (DiDonato et al., 2004). It was reported by other authors that the primary root length of the mutant *eir1-1* was longer than that of the wild type (Bao et al., 2007). In the latter case the impact of IBA (1 μM), TA-12 (1 mM) and IAA (1 μM) was particularly evident. The intense formation of lateral roots under the impact of IBA and TA-12 could be related with the ability of these compounds to intensify the mitotic activity of apical meristem cells in the lateral roots of the *Arabidopsis* allele. The present investigation revealed for the first time that IBA enhanced LR formation more effectively than IAA both in the allele and in the wild type. The question arose whether IBA acted as an auxin itself or through its conversion to IAA. Earlier investigations revealed that the metabolism and conjugation of IAA in plant tissue was more rapid than that of IBA (Novickienė and Merkys, 1998). It was shown that IBA induced adventitious roots on sterile-grown stem sections of *Arabidopsis* at concentrations where IAA was still ineffective (Ludwig-Müller, 2007). IBA was also more effective in promoting LR at lower concentrations compared to IAA in *Arabidopsis* (Zolman et al., 2000).

In summary, differences were revealed between the *Arabidopsis alf4-1* mutant and its allele phenotype in the formation of lateral roots. The impact of IBA and TA-12 on LR formation in the *alf4-1* allele could be related to the ability of these compounds to intensify the mitotic activity of apical meristem cells in the LR of the *alf4-1* allele.

From the practical point of view, the application of IBA and TA-12 leads to the formation of a larger root system, stimulating plant establishment in the soil, and improving nutrient supplies to the plant, thus ensuring better plant growth, development and productivity.

Acknowledgements

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MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES ON THE EFFECT OF SALINITY AND GROWTH PROMOTERS ON RICE PLANTS

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This study aimed to reveal changes in morphological and physiological characters during growth and mature stages of rice plants in response to salinity stress and growth promoters. Salinity stress caused a decrease in vegetative growth, yield and yield components, while growth substances enhanced the leaf area and crop yield of rice plants under salinity stress. It could be concluded that growth promoters can partially alleviate the harmful effect of salinity stress on rice.

Key words: morphological, physiological, rice, salinity, gibberellins, kinetin, urea

Introduction

Rice is one of the major field crops in Egypt. The area planted to rice is about 600,000 hectares, which is about 15% of Egypt's total cultivated area during the summer season. Efforts must be directed to overcome the serious gap between rice production and its consumption due to the huge increase in the population.

Salinity is considered to be a significant factor affecting crop production and agricultural sustainability in many regions of the world, as it reduces the value and productivity of the affected land.

In general, salinity reduces plant growth or damages the plants through (i) the osmotic effect (causing water deficit), (ii) the toxic effects of the ions and (iii) the imbalance in the uptake of essential nutrients. The cation K is essential for cell expansion, osmoregulation and cellular and whole-plant homeostasis. The role of K in response to salt stress is also well documented, where Na depresses K uptake (Fox et al., 1998). Potassium exhibited a rapid decrease in the roots, while it increased in the leaves. These results can be attributed to (i) the transfer of K from roots to leaves, (ii) the possible exchange of K ions by Na ions in root tissues, or (iii) the direct interference of Na with K uptake (Ramoliya et al., 2004; Saied et al., 2005).

The role of growth promoters in overcoming the depressing effect of salinity may be due to the enhancing effect of cytokinins, which affect the plant water balance through their effect on the stomata as well as by increasing turgor pressure (Mac Robbie, 1981) and/or decreasing root resistance to water flow. The application of GA_3 appeared to reverse the effects induced by salinity. GA_3 appears to act partially by increasing the water status of the plants and partially by sustaining metabolite levels (Sakr, 1996).

The yield and yield components of cereal grain crops may be affected by foliar urea (Emam and Borjian, 2000). It was also found that pre-anthesis foliar feeding with urea resulted in higher yields compared with later application.

The present research aimed to study the effect of GA_3, kinetin and urea on rice plants grown under different salinity levels. Growth, yields and chemical constituents were studied.

Materials and methods

Two field experiments were conducted during the summer season of 2004 and 2005 at Tag-Elezz Research Station, Dakahlia Governorate, Egypt. The experiments aimed to study the effects of gibberellins, cytokinins and urea in increasing the tolerance of rice plants to high levels of salts, the interaction between them, and the salt level that gives the best rice yield.

The rice varieties Giza 177 (salt-sensitive), Giza 178 (salt-tolerant) and Sakha 101 (semi-tolerant) were transplanted and grown on two areas with different salinity levels (EC = 4.70dS^{-1}, 6.25dS^{-1}).

Experimental design

A split-split plot design with four replications was used, with salinity levels in the main plots, varieties in the sub-plots and treatments in the sub-sub plots. The rice plants were sprayed twice, 35 and 50 days from sowing, with water (control), GA_3 (150 mg/l), kinetin (40 mg/l), GA_3 + kinetin, urea (15 %), urea + GA_3, urea + kinetin or urea + GA_3 + kinetin.

All the normal cultural practices followed by rice farmers in the district were the applied in the usual manner.

Four samples were taken during the experimental period (twice during the vegetative growth stage, i.e. 45 and 60 days after sowing, and twice during maturation growth, i.e. 115 and 125 days after transplanting).

In the vegetative growth stage, five hills were randomly chosen from each experimental plot for laboratory analysis, where the following parameters were measured: crop growth rate (CGR, g/day), relative growth rate (RGR, g/day), leaf area index (LAI), leaf area ratio and net assimilation rate (NAR, g/day) according to the formula of Charles (1982).

At harvesting, the following characters were recorded: number of filled grains/panicle, 1000-grain weight (g), grain yield (g/m^2) and grain yield (t/ha).

The data were statistically analysed using the analysis of variance (ANOVA) technique for a split plot design, according to Gomez and Gomez (1984). The treatment means were compared using the least significant difference (LSD).

Results

Physiological growth characters during vegetative growth

The data in Tables 1–5 show that GA_3 and kinetin, alone or in combination with urea, had an increasing effect on the crop growth rate, net assimilation rate, leaf area index and leaf area ratio of the rice cultivars. There was no significant difference between the salinity levels on the two experimental areas in this respect.

Table 1

Average crop growth rate (g/day) of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	0.73	0.66	0.70	0.93	0.86	0.80	1.06	0.97	1.02
GA_3	0.94	0.90	0.92	1.19	1.07	1.13	1.50	1.32	1.41
Kinetin	0.83	0.75	0.79	1.00	0.94	0.97	1.29	1.20	1.25
GA_3 + kinetin	0.84	0.82	0.83	1.04	1.02	1.03	1.22	1.23	1.23
Urea	0.88	0.75	0.82	1.01	0.95	0.98	1.17	1.08	1.13
Urea + kinetin	0.91	0.88	0.90	1.08	1.06	1.07	1.27	1.21	1.24
Urea + GA_3	1.22	1.00	1.11	1.38	1.12	1.25	1.63	1.49	1.56
Urea+kinetin+GA_3	0.96	0.90	0.93	1.03	0.96	0.99	1.10	1.00	1.05
Mean	0.91	0.83	0.88	1.08	0.99	1.03	1.28	1.19	1.14

$LSD_{5\%}$ salinity: 0.017; Variety: 0.0093; Treatment: 0.012; Salinity × variety × treatment: 0.037

Table 2

Average relative growth rate (g/day) of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	0.040	0.037	0.038	0.041	0.036	0.039	0.049	0.042	0.046
GA_3	0.044	0.042	0.043	0.046	0.044	0.045	0.058	0.047	0.053
Kinetin	0.042	0.036	0.039	0.042	0.043	0.043	0.054	0.044	0.049
GA_3 + kinetin	0.043	0.037	0.040	0.042	0.042	0.042	0.050	0.045	0.048
Urea	0.041	0.040	0.041	0.043	0.041	0.042	0.053	0.043	0.048
Urea + kinetin	0.044	0.039	0.042	0.045	0.045	0.045	0.052	0.045	0.049
Urea + GA_3	0.046	0.044	0.045	0.050	0.047	0.049	0.060	0.049	0.055
Urea+kinetin+GA_3	0.041	0.039	0.040	0.045	0.042	0.044	0.056	0.045	0.051
Mean	0.043	0.039	0.041	0.044	0.43	0.044	0.054	0.045	0.050

$LSD_{5\%}$ salinity: 0.001; Variety: 0.009; Treatment: 0.0011; Salinity × variety × treatment: 0.004

The interaction between GA_3, kinetin or urea with salinity levels led to an increase in crop growth rate, net assimilation rate, leaf area index and leaf area ratio in all three rice cultivars on the two experimental areas (area 1 and area 2).

The GA_3 + urea treatment was most effective in counteracting the harmful effect of salinity stress on the different rice cultivars (sensitive, semi-tolerant and tolerant).

Table 3

Average leaf area index of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels at two sampling dates in the 2004 and 2005 seasons

A. 45 days from sowing

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	3.0	2.5	2.75	3.1	2.5	2.80	3.1	2.7	2.90
GA_3	3.4	3.0	3.20	3.4	3.3	3.35	3.6	3.5	3.55
Kinetin	3.1	2.9	3.00	3.2	2.9	3.05	3.2	3.0	3.10
GA_3 +kinetin	3.2	2.7	2.95	3.2	2.5	2.85	3.3	2.9	3.10
Urea	3.2	2.8	3.00	3.2	2.7	2.95	3.3	3.0	3.15
Urea + kinetin	3.1	2.6	2.85	3.3	2.7	3.00	3.3	3.3	3.30
Urea + GA_3	3.5	3.3	3.40	3.6	3.4	3.50	3.8	3.5	3.65
Urea+kinetin+GA_3	3.3	3.1	3.20	3.2	3.0	3.10	3.6	3.3	3.45
Mean	3.23	2.86	3.04	3.28	2.88	3.08	3.4	3.15	3.28

$LSD_{5\%}$ salinity: 0.12; Variety: 0.106; Treatment: 0.14; Salinity × variety × treatment: 0.41

B. 60 days from sowing

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	3.6	3.3	3.45	3.7	3.4	3.55	3.9	3.7	3.80
GA_3	4.2	4.0	4.10	4.3	3.9	4.10	4.4	4.0	4.20
Kinetin	3.9	3.7	3.80	3.9	3.8	3.85	4.2	3.8	4.00
GA_3 + kinetin	3.7	3.4	3.55	4.0	3.6	3.80	4.1	3.9	4.00
Urea	3.9	3.8	3.85	4.0	3.6	3.80	4.1	3.9	4.00
Urea + kinetin	3.8	3.6	3.70	3.8	3.7	3.75	4.2	4.0	4.10
Urea + GA_3	4.4	4.0	4.20	4.5	4.2	4.35	4.6	4.3	4.45
Urea+kinetin+GA_3	4.0	3.9	3.95	4.2	3.8	4.00	4.4	4.0	4.20
Mean	3.94	3.71	3.83	4.05	3.75	3.90	4.21	3.95	4.09

$LSD_{5\%}$ salinity: 0.82; Variety: 0.2; Treatment: 0.26; Salinity × variety × treatment: 0.55

Table 4

Average leaf area ratio of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (Salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels in 2004 and 2005

A. 45 days from sowing

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	152.8	135.6	144.2	148.5	132.4	140.4	140.2	129.9	135.1
GA_3	165.8	147.8	156.8	155.6	139.4	147.5	146.5	134.9	140.7
Kinetin	172.1	154.1	163.1	163.5	145.5	154.5	150.1	136.5	143.3
GA_3 + kinetin	154.6	137.8	146.2	151.6	134.5	143.1	142.3	131.1	136.7
Urea	159.6	143.6	151.6	154.4	137.7	146.0	144.8	133.8	139.3
Urea + kinetin	156.7	141.9	149.3	153.3	136.5	144.9	143.2	133.2	138.2
Urea + GA_3	178.7	163.2	170.9	168.8	148.6	158.7	154.6	139.5	147.0
Urea+kinetin+GA_3	166.5	151.6	159.0	157.4	141.5	149.4	148.3	135.6	141.9
Mean	163.3	146.9	155.1	156.6	139.5	148.1	146.2	134.3	140.2

$LSD_{5\%}$ salinity: 0.017; Variety: 0.063; Treatment: 0.08; Salinity × variety × treatment: 0.32

B. 60 days from sowing

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	142.3	117.5	129.9	138.5	115.2	126.8	136.4	113.2	124.8
GA_3	147.8	120.3	134.0	142.3	120.3	131.3	138.2	115.3	126.7
Kinetin	149.3	121.9	135.6	143.4	121.3	132.3	138.9	116.1	127.5
GA_3 + kinetin	143.9	118.3	131.1	139.6	117.3	128.4	137.3	113.8	125.5
Urea	146.8	119.5	133.1	141.3	119.7	130.5	138.4	114.9	126.6
Urea + kinetin	144.5	118.9	131.7	140.2	118.8	129.5	137.9	114.2	126.0
Urea + GA_3	151.3	123.2	137.2	144.6	122.3	133.4	139.3	116.9	128.1
Urea + kinetin + GA_3	148.2	120.4	134.3	142.6	120.9	131.7	138.5	115.6	127.0
Mean	146.7	120.0	133.3	141.5	119.4	130.5	138.1	115.0	126.5

$LSD_{5\%}$ salinity: 0.096; Variety: 0.095; Treatment: 0.12; Salinity × variety × treatment: 0.39

Table 5

Average net assimilation rate (g/day) of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	1.15	1.08	1.12	1.21	1.18	1.12	1.23	1.19	1.12
GA_3	1.34	1.28	1.31	1.38	1.34	1.36	1.35	1.32	1.34
Kinetin	1.17	1.18	1.18	1.27	1.26	1.27	1.24	1.31	1.28
GA_3 + kinetin	1.28	1.21	1.25	1.26	1.22	1.24	1.23	1.31	1.27
Urea	1.29	1.21	1.25	1.31	1.30	1.31	1.28	1.23	1.26
Urea + kinetin	1.19	1.23	1.21	1.25	1.28	1.27	1.30	1.23	1.27
Urea + GA_3	1.40	1.35	1.38	1.43	1.36	1.40	1.59	1.38	1.49
Urea + kinetin + GA_3	1.29	1.20	1.25	1.36	1.26	1.31	1.39	1.28	1.34
Mean	1.26	1.22	1.24	1.31	1.28	1.29	1.32	1.28	1.30

$LSD_{5\%}$ salinity: 0.034, Variety: 0.033; Treatment: 0.043; Salinity × variety × treatment: 0.11

Yield and yield components

The data in Tables 6–9 show that the number of filled grains/panicle, 1000-grain weight and grain yield of different rice cultivars were greatly affected by salinity levels. The sensitive rice cultivar was more affected by salinity stress, while the tolerant cultivars were less affected. With the exception of kinetin alone, all the growth substances, urea and their combinations increased these parameters, especially at the first sampling date (115 days from sowing). GA_3 alone or GA_3 + urea at the second sampling date (125 days from sowing).

The interaction of salinity stress levels with growth substances was not significant.

Table 6

Average number of filled grains/panicle of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

A. 115 days from sowing

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	91.6	80.6	86.1	97.3	91.0	94.1	105.0	102.3	103.6
GA_3	111.7	99.0	105.3	118.3	112.3	115.3	122.0	115.0	118.5
Kinetin	92.3	82.6	87.4	99.3	92.3	95.8	106.3	103.3	104.8
GA_3 + kinetin	96.3	88.3	92.3	107.6	99.3	103.4	110.0	109.7	109.8
Urea	99.0	92.6	95.8	109.0	100.0	104.5	112.3	106.3	109.3
Urea + kinetin	94.6	85.3	89.9	102.6	95.7	99.15	108.3	105.0	106.6
Urea + GA_3	117.0	107.3	112.1	123.6	119.3	121.4	127.6	122.0	124.8
Urea + kinetin + GA_3	109.7	96.0	102.8	110.3	101.6	105.9	114.6	108.3	111.4
Mean	101.5	91.4	96.4	108.5	101.4	104.9	113.26	108.9	111.1

$LSD_{5\%}$ salinity: 7.03; Variety: 7.1, Treatment: 9.07; Salinity × variety × treatment: NS

B. 125 days from sowing

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	94.6	83.6	89.1	99.3	95.0	97.1	109.0	105.3	107.1
GA_3	114.7	102.0	108.3	120.3	116.3	118.3	125.0	117.0	121.0
Kinetin	96.3	85.6	90.9	101.3	95.3	98.3	108.3	105.3	106.8
GA_3 + kinetin	99.3	92.3	95.8	109.6	101.3	105.4	113.0	111.7	112.3
Urea	102.0	96.6	99.3	112.0	104.0	108.0	116.3	108.3	112.3
Urea + kinetin	97.6	88.3	92.9	105.6	99.7	102.6	110.3	108.0	109.1
Urea + GA_3	119.0	110.3	114.6	126.6	122.3	124.4	129.6	125.0	127.3
Urea + kinetin + GA_3	111.7	99.0	105.3	114.3	104.6	109.4	116.6	110.3	113.4
Mean	104.4	94.7	99.5	111.1	104.8	107.9	116.0	111.3	113.6

$LSD_{5\%}$ salinity: 9.46; Variety: 5.89; Treatment: 7.53; Salinity × variety × treatment: NS

Table 7

Average 1000 grain weight (g) of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	21.2	15.5	18.35	23.5	22.3	22.9	23.1	21.0	22.05
Urea	23.6	17.5	20.55	26.0	24.2	25.1	24.8	22.6	23.7
GA_3	24.4	18.5	21.45	28.2	25.2	26.7	25.6	23.9	24.75
Kinetin	21.4	15.7	18.55	23.9	22.5	23.2	23.3	21.1	22.2
GA_3 + kinetin	23.0	16.5	19.75	24.5	23.3	23.9	24.3	22.4	23.35
Urea + kinetin	22.5	16.1	19.3	24.2	23.0	23.6	23.6	21.7	22.65
Urea + GA_3	25.0	19.7	22.35	29.1	26.2	27.65	26.7	24.5	25.6
Urea + kinetin + GA_3	24.0	18.5	21.25	27.0	24.5	25.75	24.3	23.2	23.75
Mean	23.14	17.25	20.19	25.8	23.9	24.85	24.46	22.55	23.06

$LSD_{5\%}$ salinity: 0.21; Variety: 0.37; Treatment: 0.48; Salinity × variety × treatment: 0.95

Table 8

Average yield (g/m^2) of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	762	683	722.5	978	705	796.5	995	753	874
Urea	815	756	785.5	1012	748	880	1027	808	917.5
GA_3	846	767	806.5	1021	814	917.5	1039	935	987
Kinetin	771	692	731.5	984	716	850	1002	774	888
GA_3 + kinetin	797	708	752.5	1009	742	875.5	1025	792	908.5
Urea + kinetin	785	702	743.5	997	723	860	1013	785	899
Urea + GA_3	855	781	818	1032	792	912	1052	987	1019.5
Urea + kinetin + GA_3	813	740	776.5	1013	732	872.5	1032	917	974.5
Mean	805.5	728.6	767.1	1005.8	741.5	870.5	1023.13	843.9	933.5

$LSD_{5\%}$ salinity: 161; Variety: 137; Treatment: 75; Salinity × variety × treatment: 495

Table 9

Average yield (t/ha) of rice cultivars Giza 177 (salt-sensitive), Sakha 101 (semi-tolerant) and Giza 178 (salt-tolerant) as affected by different concentrations of urea, GA_3 and kinetin at two salinity levels during the 2004 and 2005 seasons

Treatments	Giza 177			Sakha 101			Giza 178		
	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean	Ec =4.7	Ec= 6.25	Mean
Control	7.62	6.83	7.21	8.95	7.05	7.10	9.94	7.52	8.73
Urea	8.14	7.57	7.85	9.19	7.49	8.35	10.26	8.21	9.23
GA_3	8.45	7.74	8.09	9.38	7.66	8.52	10.37	9.21	9.81
Kinetin	7.71	6.93	7.33	9.02	7.16	8.09	10.02	7.74	8.88
GA_3 + kinetin	7.97	7.26	7.62	9.14	7.43	8.28	10.13	7.91	9.02
Urea + kinetin	7.85	7.02	7.45	9.07	7.26	8.52	10.06	7.81	8.95
Urea + GA_3	8.62	7.91	8.23	9.47	7.90	8.68	10.52	9.43	9.97
Urea + kinetin + GA_3	8.14	7.41	7.78	9.31	7.43	8.38	10.31	9.09	9.71
Mean	8.11	7.31	7.69	9.28	7.43	8.35	10.21	8.35	9.28

$LSD_{5\%}$ salinity: 0.031; Variety: 0.028; Treatment: 0.032; Salinity × variety × treatment: 0.11

Discussion

Effect of salinity stress on growth

Alscher et al. (1997), Lin and Kao (1995), Hernandez et al. (2000) and Khan and Panda (2002) attributed the depressing effects of salt stress on plant growth to (1) an increase in reactive oxygen species, which play an important role in DNA damage as well as in damage to all classes of biologically important macromolecules, (2) the generation of potentially toxic reactive oxygen species such as H_2O_2 and lipid hydroperoxides, which cause membrane changes, (3) the accumulation of antioxidant enzymes in root tissues, and (4) an increase in the activity of ascorbate peroxidase (APX), glutathione reductase (GR), monohydroascorbate reductase (MDHAR), super oxide dismutase (SOD) and dehydroascorbate reductase (DHAR), especially in tolerant rice plants, while in sensitive rice plants salinity decreased the super oxide dismutase (SOD) activity by about 35%.

Schwarz and Gale (1981), Imamul-Huq and Lather (1983), Sakr (1996) and Ozdemir et al. (2004) reported that the role of growth substances (kinetin, GA_3, IAA and ethrel) in overcoming the depressing effect of salinity stress on growth and biochemical constituents may be due to one or more of the following: (a) increasing root dry weight and decreasing root resistance to water flow and Cl^- uptake, (b) nullifying changes in mineral composition, photosynthetic pigments and endogenous hormonal levels, (c) increasing the water absorption capacity of the roots, (d) increasing K^+ uptake, sugar accumulation and oligosaccharide content within plant tissues, (e) increasing water status within the plant tissues by sustaining metabolite levels including K^+ over Na^+, and increasing organic acid contents and ion uptake, (f) increasing the stimulator/inhibitor ratio in the plant tissues, (g) increasing the content of carbohydrates, proline and organic acids, which can be used as an indicator in the osmoregulation of tissues under salinity stress.

The data presented in the tables show the enhancing effect of urea on rice growth under salinity stress.

The fertilization of plants is important to minimize the hazardous effect of salinity on growth. Marschner (1995) suggested that increased fertilization might overcome some of the inhibitory effects of salinity. The interactions between soil fertility and cultivated crops are of major interest in optimizing crop production under salinity conditions (Hanafy et al., 2002).

The increase in growth parameters in response to nitrogen might be explained by the increased production of auxin in the plants, presumably due to an increase in tryptophan, the precursor of IAA. It also appears that the increase in endogenous growth hormones due to improved N supplies might overcome the effect of salt stress in reducing the synthesis of these hormones, which control several metabolic processes in plants (Hanafy et al., 2002). In addition, the antagonism between N and both Cl^- and Na^+ could lead to the higher absorption of nutrients at the expense of Cl^- and Na^+, resulting in the greater incorporation of N in proteins and higher yield.

Yield and yield components

Salinity affects all stages of growth and development, as well as the yield of rice. The seed yield is more severely depressed by salt than the vegetative growth. The reduction in seed yield is largely due to a decrease in seed set, which may be attributed to the poorer viability of the pollen or the reduced receptivity of the stigmatic surface, or both (Sakr et al., 2004).

The reduction in pollen viability has been attributed to the decreased calcium mobilization from plant leaves treated with sodium chloride, which is important for pollen germination and pollen tube growth. There is also a significant reduction in grain number due to the substantial abscission of flowers or young grain due to ethylene induction by salinity. Factors affecting cell division and cell expansion, such as tissue water status and the concentration of

certain plant hormones (e.g.. ABA) are involved in the regulation of seed set under stress. It has also been revealed that increasing salinity levels significantly decreased yield due to reductions in pollen grain production, mean number of perfect flowers and fruit set. The depressing effect of salinity on yield may be due to a decrease in the leaf area and number per plant, resulting in a lower supply of carbon assimilates due to a decrease in the net photosynthetic rate and biomass accumulation. The detrimental effects of salinity stress on growth characters and yield might be partially due to a decrease in nitrogen concentration.

The data show that salinity stress decreased many parameters such as number of tillers, leaf area index, accumulation of dry matter, photosynthetic pigments, nitrogen and phosphorus, as well as potassium uptake and the carbohydrate and sugar contents. This was reflected in lower yields and yield components.

Role of urea in counteracting the harmful effect of salinity stress on yield and yield components

It could be concluded from the results that urea nullified the reduction observed in rice yield due to salinity stress by enhancing growth (tiller number, leaf area index and dry matter accumulation).

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SALT TOLERANCE OF TWELVE MAIZE HYBRIDS AT THE SEEDLING STAGE

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Using a simple and novel semi-hydroponic technique, three experiments were carried out to evaluate salinity tolerance in twelve pipe-line/commercial maize hybrids at the seedling stage. For experiment 1, hybrids were evaluated at 0.10, 0.15 and 0.20 M NaCl. For experiments 2 and 3, hybrids were subjected to 0.25 and 0.30 M NaCl, respectively. The technique simulates a semi-hydroponic system where the upper layers of coco peat medium receive water/or saline solution only by capillary movement, while the roots are immersed in saturated lower coco peat medium. The emergence percentage ranged from 73 to 100% under control conditions, from 50 to 100% at 0.10 M NaCl, from 46 to 100% at 0.15 M NaCl and from 43 to 96% at 0.20 M NaCl. In addition, increasing salinity decreased the shoot and the root length and the seedling dry weight. Several hybrids (VMH 4029, VMH 4028, VMH 4088, VMH 4060, VMH 4033, VMH 4046, VMH 4101) exhibited a high emergence percentage of 80–96% even at 0.3 M NaCl, revealing that these hybrids have high salinity tolerance and also the capacity to produce high yields. This confirms that salinity tolerance and high yield may be combined if pipe-line hybrids which have been confirmed for high yield over multilocation trials are selected and then tested under field conditions in saline-prone areas.

Key words: maize hybrids, salinity tolerance, seedling stage, emergence, shoot, root length

Introduction

Maize (*Zea mays* L.) is grown under diverse climatic conditions in various countries of the world. It is an important basic food grain, particularly for the population of Latin American countries. It occupies an indispensable place in the human diet due to its high nutritional quality, but the productivity of the crop is affected by several abiotic factors such as cold temperature, drought and salinity (Maiti et al., 2004). Therefore, there is a great necessity to develop an efficient technique for the evaluation and selection of maize cultivars with tolerance of salinity as well as other abiotic stresses. Significant progress has been achieved in the salinity resistance of maize.

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Salt stress affects the growth of a crop at different stages. Genotypic variability exists for resistance to salinity (Nordquist et al., 1992). Maiti et al. (1996) reported high genetic variability in maize cultivars (*Zea mays*) for resistance to drought and salinity at the seedling stage, and diverse genotypes were selected for resistance to drought and salinity stress. Increasing salinity reduces the germination rate (Jan et al., 1995), but the addition of $CaCl_2$ improves seedling growth in terms of shoot and root dry weight (Alberico and Cramer, 1993). Salt shock inhibits root elongation, but it is followed by a gradual recovery (Rodriguez et al., 1997). Several studies have been undertaken to understand the mechanism of tolerance to salinity in maize and other crops (Giaveno et al., 2007). The plasma membrane may be the primary site of salt injury (Läuchli, 1990; Mansour, 1997).

Plasma membrane permeability is altered markedly by salt exposure in salt-sensitive cultivars, whereas the effect is always marginal in salt-tolerant cultivars (Mansour and Salama, 2004). In the context of the review by Maiti et al. (2004), it has been assessed that the mechanism of resistance of maize cultivars to salinity involves three principal phenomena: 1) ion accumulation, 2) osmotic adjustment and 3) irregular metabolism. Thus, the present study was carried out with the aim of quantifying and comparing the growth responses of twelve maize hybrids to different levels of NaCl as an approach to selecting genetic material and identifying seedling traits indicative of salt stress tolerance.

Materials and methods

Plant material and experimental conditions

The study was conducted at the Seed Physiology Laboratory, Vibha Agrotech Ltd. (VAL), Hyderabad, India (July to September, 2008) using different sets of maize hybrids developed by VAL for tolerance to NaCl salinity at the seedling stage. Three experiments were conducted with different sets of maize hybrids and their parents for this purpose.

Twelve maize hybrids (Elite, Legend, Boom, VMH-4028, -4029, -4033, -4040, -4046, -4060, -4088, -4092 and -4101) developed at VAL were grown in plastic pots using sterilized coco peat (coir peat) at room temperature (about 27°C) under artificial light. The novel technique developed for this purpose consists of filling plastic pots (height 7.5 cm, diameter 5 cm) with coco peat (neutral delignified coir fibres) and then applying water or the required saline concentration (0.10–0.30 M NaCl) up to two-thirds of the pot (about 70 mm) on a single occasion. Twenty seeds were sown in each pot at a depth of 2 cm in the upper coco peat layer, which receives water/salt solution by capillarity. To protect the seeds from fungal attack, they were treated with thiram solution (5% v/v) for 5 minutes before sowing. Each of the treatments was replicated thrice for all the hybrids and the experiment was terminated 10 days after emergence. This technique simulates a semi-hydroponic system where the upper layers of coco peat medium receive water or saline solution only by capillary movement, while the roots are immersed in the lower saturated coco peat medium. During capillary movement there is a free flow of oxygen owing to the constant evapo-transpiration. Observations on the average emergence percentage, shoot length (cm), root length (cm) and dry weight (mg) were made on five randomly sampled seedlings on the 10th day after emergence. The same procedure and the same variables were used in all the experiments. The main objective of the present study was to determine the efficacy of the new technique on different sets of maize hybrids for the evaluation of tolerance of NaCl salinity. In experiment 1, the maize

hybrids were evaluated at 0.10, 0.15 and 0.20 M NaCl, while in experiments 2 and 3, they were subjected to 0.25 and 0.30 M NaCl, respectively. In each experiment, along with the distilled water (control) or the relevant NaCl concentration, sufficient nutrient solution (Knop's solution) was added to supply the plant nutrients required for growth.

Statistical analyses

Data of the seedling traits were analysed statistically for each experiment using one-way analysis of variance with a factorial arrangement, hybrids and NaCl concentrations being the factors. Where the F-test was significant ($p<0.05$), the differences were validated using Tukey's test. Assumptions on the normality of the data were tested using the Kolmogorov–Smirnov test (Steel and Torrie, 1980). All the statistical methods applied were computed using the SPSS® (Statistical Package for the Social Sciences) software package (standard released version 13.0 for Windows, SPSS Inc., Chicago, IL).

Results

Experiment 1

With the exception of the hybrid × NaCl concentration interaction for the emergence percentage, all the seedling traits exhibited significant differences between maize hybrids and NaCl concentrations, and between hybrids within the NaCl concentrations (Table 1). The emergence percentage ranged from 73% (Legend) to 100% (VMH-4046, -4060, -4088 and -4101) under control conditions, from 50% (Legend) to 100% (VMH-4046, -4088, -4101) at 0.10 M NaCl, from 46% (Legend) to 100% (VMH-4046) at 0.15 M NaCl, and from 43% (Legend) to 96% (VMH-4046 and -4088) at 0.20 M NaCl (Fig. 1a). The shoot length ranged from 11.9 cm (VMH-4092) to 24.9 cm (VMH-4046) under control conditions, from 7.2 (VMH-4092) to 17.6 cm (VMH-4046) at 0.10 M NaCl, from 8.5 cm (Legend) to 15.8 cm (VMH-4046) at 0.15 M NaCl, and from 7.0 (VMH-4040) to 12.8 cm (VMH-4046) at 0.20 M NaCl (Fig. 1b). The root length varied from 16.8 cm (VMH-4029) to 24.4 cm (Boom) under control conditions, from 11.4 cm (Legend) to 23.9 cm (Boom) at 0.10 M NaCl, from 9.7 cm (Legend) to 20.1 cm (Boom) at 0.15 M NaCl, and from 9.5 cm (Legend) to 14.7 cm (VMH-4046) at 0.20 M NaCl (Fig. 1c). Seedling dry weight varied from 423 mg (VMH-4040) to 776 mg (VMH-4060) under control conditions, from 366 mg (Legend) to 656 mg (VMH-4029) at 0.10 NaCl, from 226 mg (Legend) to 576 mg (VMH-4029) at 0.15 M NaCl, and from 193 mg (Legend) to 540 mg (VMH-4029) at 0.20 M NaCl (Fig. 1d). As a general trend, the emergence percentage, shoot and root length and seedling dry weight of the maize hybrids decreased by about 21, 44, 37 and 32%, respectively, between the control and 0.20 M NaCl.

Experiment 2

The results of this experiment revealed that there were highly significant differences between the maize hybrids, the NaCl concentrations and between hybrids within each NaCl concentration for the studied seedling traits (Table 1). Under control conditions, VMH-4029, -4046, -4060, -4088 and -4101 achieved

100% emergence, in contrast to a value of 73% for Legend, while at 0.25 M NaCl it ranged from 23% (Legend) to 93.3% (VMH-4046) (Fig. 2a). Shoot length ranged from 12.6 cm (VMH-4092) to 24.9 cm (VMH-4088) under control conditions, whereas at 0.25 M NaCl it varied from 7.9 cm (VMH-4092) to 15.6 cm (VMH-4046) (Fig. 2b). The lowest (16.7 cm) and highest (24.0 cm) values of root length were detected in VMH-4029 and Boom, respectively, under control conditions, while at 0.25 M NaCl, it ranged from 7.1 cm (Legend) to 13.6 cm (VMH-4060) (Fig. 2c). Seedling dry weight ranged from 480 mg (VMH-4092) to 796 mg (VMH-4040) under control conditions and from 156 mg (Legend) to 640 mg (VMH-4040) at 0.25 M NaCl (Fig. 2d). As a general pattern, the emergence percentage, shoot and root length and seedling dry weight of maize hybrids decreased by about 26, 37, 47 and 30%, respectively, at 0.25 M NaCl compared with the control.

Table 1

Calculated mean squares (MS) and F values from the statistical analysis on the data of twelve maize hybrids subjected to 0.1, 0.15 and 0.2 M NaCl (Experiment 1), 0.25 M NaCl (Experiment 2) and 0.30 M NaCl (Experiment 3). Each experiment included a control

Experimental conditions	Source of variation	Seedling trait							
		Emergence		Shoot length		Root length		Seedling dry weight	
		MS	F	MS	F	MS	F	MS	F
Experiment 1	Hybrids (H)	2298.2	18.7^{***}	96.7	34.6^{***}	49.5	17.0^{***}	62816.9	7.1^{***}
	NaCl	2571.3	20.9^{***}	442.7	158.6^{***}	334.1	114.6^{***}	245356.3	27.7^{***}
	H × NaCl	123.3	1.0^{NS}	6.5	2.3^{***}	10.5	3.6^{***}	15722.9	1.7^{*}
	Error	122.9		2.7		2.9		8832.6	
	Mean	84.0		13.2		15.5		472.7	
	r^2	0.640		0.861		0.808		0.548	
	CV (%)	13.2		12.6		10.9		19.8	
Experiment 2	Hybrids (H)	1081.5	112.2^{***}	49.4	39.7^{***}	14.2	7.5^{***}	73803.0	36.2^{***}
	NaCl	10370.8	1075.5^{***}	836.3	672.0^{***}	1559.6	819.8^{***}	549676.1	269.7^{***}
	H × NaCl	253.2	26.3^{***}	17.5	14.1^{***}	11.8	6.2^{***}	10547.0	5.1^{***}
	Error	9.6		1.2		1.9		2037.4	
	Mean	80.4		14.9		14.9		488.8	
	r^2	0.974		0.946		0.930		0.908	
	CV (%)	3.9		7.4		9.2		9.2	
Experiment 3	Hybrids (H)	516.0	31.7^{***}	10.4	1.7^{NS}	7.2	1.3^{NS}	89382.3	65.5^{***}
	NaCl	1134.9	39.8^{***}	1229.3	196.4^{***}	1972.0	363.1^{***}	104253.5	76.4^{**}
	H × NaCl	183.7	11.3^{***}	24.2	3.9^{**}	11.2	2.1^{NS}	5559.0	4.0^{**}
	Error	16.3		6.3		5.4		1363.5	
	Mean	89.5		16.8		17.4		551.6	
	r^2	0.883		0.824		0.888		0.921	
	CV (%)	4.5		14.8		13.3		6.7	

^{NS}Not significant, $p>0.05$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; r^2: adjusted coefficient of determination; cv: coefficient of variation

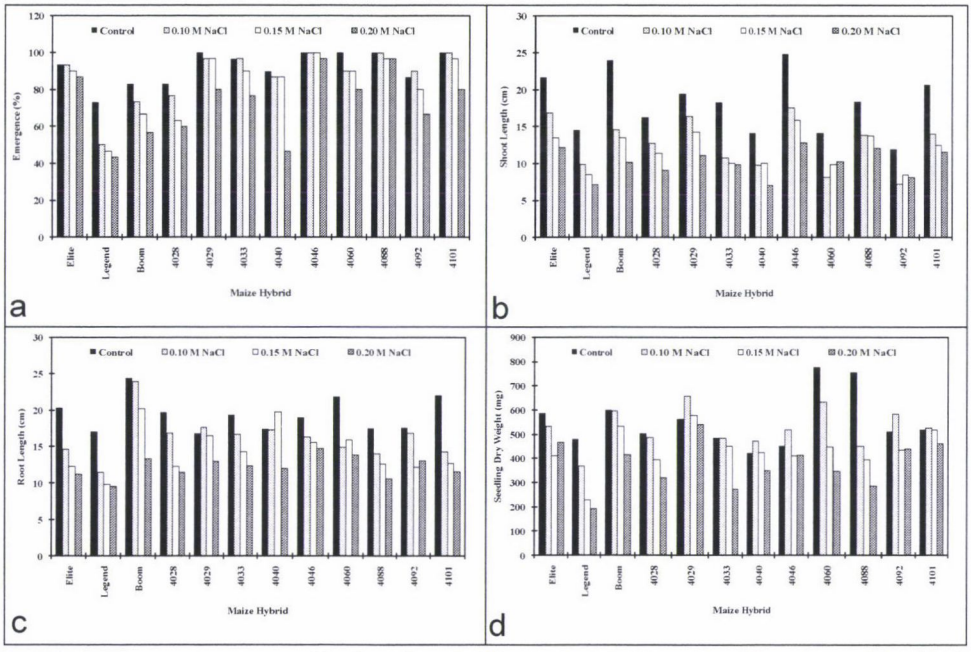

Fig. 1. Emergence percentage (a), shoot length (b), root length (c) and seedling dry weight (d) of twelve maize hybrids grown under control conditions (water+Knop's nutrient solution) or at 0.10, 0.15 and 0.20 M NaCl

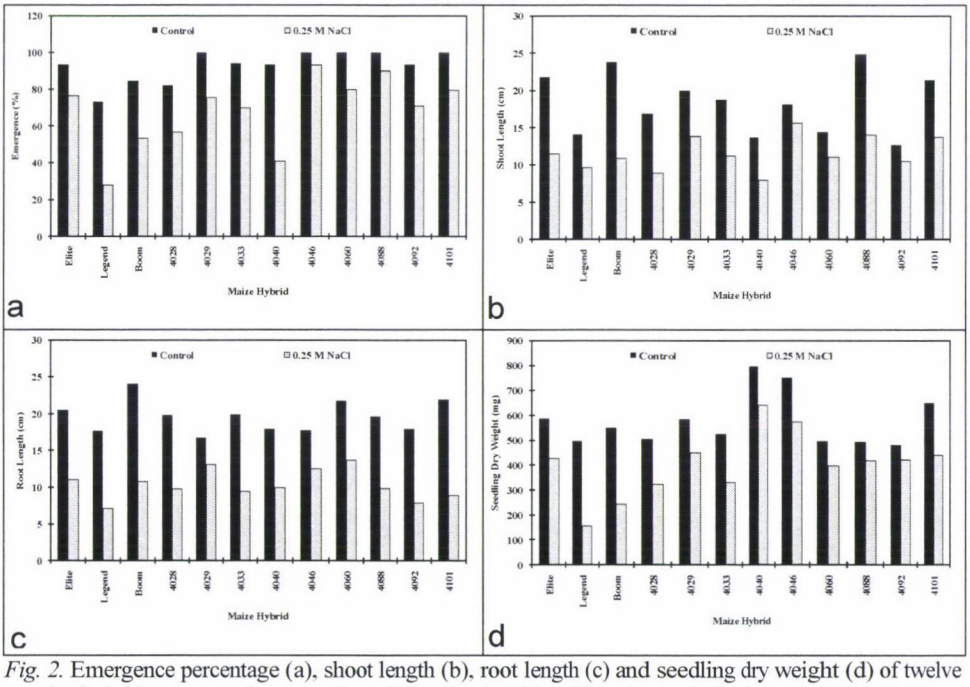

Fig. 2. Emergence percentage (a), shoot length (b), root length (c) and seedling dry weight (d) of twelve maize hybrids grown under control conditions (water+Knop's nutrient solution) or at 0.25 M NaCl

Experiment 3

One-way analysis of variance detected highly significant differences between maize hybrids, NaCl concentrations, and hybrids within NaCl concentrations for emergence percentage and seedling dry weight. In addition, differences were also revealed between NaCl concentrations for shoot and root length, and for the hybrid × NaCl concentration interaction for shoot length (Table 1). The emergence percentage ranged from 83% (VMH-4028) to 100% (Elite) under control conditions, and from 53% (VMH-4028) to 96% (VMH-4060) at 0.3 M NaCl (Fig. 3a). The maximum (25.9 cm) and minimum (9.53 cm) values of shoot length were detected in VMH-4046 under control conditions and at 0.3 M NaCl, respectively (Fig. 3b). The mean values of root length were 23.8 cm and 11.0 cm in the control and at 0.3 M NaCl, respectively (Fig. 3c). Seedling dry weight ranged from 471 mg (VMH-4033) to 761 mg (VMH-4088) under control conditions and from 346 mg (VMH-4033) to 705 mg (VMH-4088) at 0.3 M NaCl (Fig. 3d). In general, the emergence percentage, shoot and root length and seedling dry weight of maize hybrids decreased by about 10, 46, 53 and 15%, respectively, at 0.30 M NaCl compared with the control.

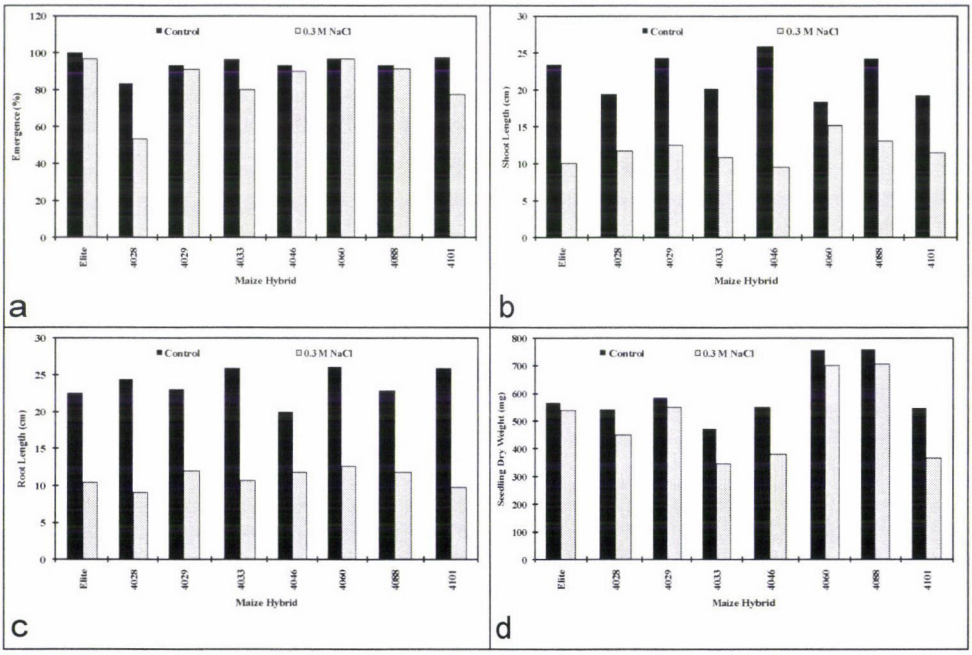

Fig. 3. Emergence percentage (a), shoot length (b), root length (c) and seedling dry weight (d) of eight maize hybrids grown under control conditions (water+Knop's nutrient solution) or at 0.30 M NaCl

Discussion

The results of the three experiments suggested the existence of genetic variability between maize hybrids, which could be selected for the improvement of salt tolerance during the early stages of crop growth and development. The results also indicated that of the seedling traits studied, the emergence percentage and seedling dry weight could be used as salt stress selection criteria for concentrations above 0.25 M NaCl. Among the maize hybrids studied, it seems that the most promising genotypes for tolerance of concentrations up to 0.3 M NaCl are Elite, VMH-4060, -4088, -4029 and -4046 in terms of emergence (values above 90%), and VMH-4060 and -4088 based on seedling dry weight. In contrast, the most susceptible hybrids in terms of both emergence and seedling dry weight were VMH-4028, -4033, -4101 and -4046. Thus, for mass genetic screening selection, materials could be evaluated up to 0.3 M NaCl in order to identify sources of germplasm tolerant of salt stress.

The present study showed high genetic variability in salinity tolerance with respect to different seedling traits (emergence percentage, shoot length, root length and dry weight in different experiments at different levels of salinity). The results of the present study are in agreement with the results achieved by Giaveno et al. (2007) in screening tropical maize for salt tolerance. Genotypic variability was also reported by Maiti et al. (1996). Several hybrids with emergence percentages of up to 90% have been selected from different experiments. Increasing salinity caused a decrease in emergence percentage, shoot length, root length and seedling dry weight, as also observed by Rodriguez et al. (1997).

The adaptation of embryonic cultures to salinity stress is reported to be associated with qualitative and quantitative changes in the polypeptide pattern (Lusardi et al., 1991). Tolerant lines accumulate glycinebetain (GB), which reduces shoot growth inhibition under saline conditions and helps to maintain greater relative leaf water content, a higher rate of carbon assimilation, and greater turgor potential (Saneoka et al., 1995). The heritability for relative root growth is moderate, suggesting that there is good scope for enhancing salt tolerance in maize through selection and breeding (Khan et al., 2003). Salinity affects water transport in maize roots (Azaizeth and Steudle, 1991). The mechanism of Na exclusion and Na inclusion functions as a tolerance mechanism in maize (Cramer, 1992). The growth and mineral absorption of maize seedlings is affected by increasing NaCl (Izzo et al., 1991). Jan (1999) studied the salt tolerance of maize cultivars under salt-stressed conditions and observed that, though salinity reduced root elongation, it increased the production of profuse lateral fine roots, possibly due to an increase in osmotic adjustment.

Seven hybrids (VMH-4029, -4028, -4088, 4060, -4033, -4046, and -4101) showed high emergence percentages of 80–96% even at the highest stress. These hybrids are well adapted to a wide range of agro-climatic conditions and have high yielding capacity, so they are suitable for testing in field conditions under saline-prone areas. It is suggested that the evaluation and selection of pipe-line hybrids could offer great scope for the combination of high yield and stress resistance (salinity/drought). This is considered as an effective approach for increasing productivity under salinity stress. Traits associated with seedling vigour, such as seedling weight and growth rate, and photochemical efficiency under stress conditions can be used as selection criteria for salt-tolerant maize in breeding programmes (Giaveno et al., 2007).

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INFLUENCE OF INCREASING SEED OIL CONTENT ON THE FATTY ACID PROFILE OF HEMP (*Cannabis sativa* L.)

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This paper reports changes in the fatty acid profiles of the hemp seed oil of two breeding populations, Kolaj and Fibrol, after five and seven years of selection for high oil content. While the original variety Kompolti had a 3:1 ratio of linoleic acid to linolenic acid, which has been claimed to be optimal for human nutrition, the selected population of Kolaj (improved Kompolti) shows a ratio close to 4:1.

The highest ratio of increase (r.i.) was 1.45, determined for γ-linolenic acid in Fibrol (improved Fibrimon 21-63). Another significant change was the 0.49% (r.i. 1.21) increase in stearic acid in this variety, along with a 3.16% (r.i. 1.1) increase in total oil content. In Kolaj the 5.87% (r.i. 1.2) increase in oil content was accompanied by a 1.76 % (r.i. –1.28) decrease in palmitic acid and by decreases of 2.98% and 0.18% (r.i. –1.15 and –1.16), respectively, in the α- and γ-linolenic acid contents.

Key words: Fibrol, Kolaj, oil content, changes in fatty acid ratio, monoecious, dioecious, ratio of LA and LNA

Introduction

The cold-pressed hemp seed oil market has been growing rapidly in the last few years, as has the number of companies offering it for human consumption as a dietary supplement and/or for medical and cosmetic purposes. Its fatty acid composition is unique among common plant oils for at least two reasons:

1) Its high content of polyunsaturated fatty acids, which may exceed 80% (Erasmus, 1993; Kralovánszky and Marthné Schill, 1994)

2) The optimal 3 : 1 ratio of linoleic (LA) to linolenic (LNA) acids, which are essential fatty acids in the human diet (Erasmus, 1993).

Due to the high γ-linolenic acid (GLA) content, its oil, like that of evening primrose, black currant, borago and *Spirulina*, is frequently recommended for

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the treatment of atopic excema and mastalgia (Deferne and Pate, 1996). Its beneficial effects in cardiovascular, psychiatric and immunological disorders have also been investigated (Horrobin, 1990a; 1990b; 1992).

Because of its high level of polyunsaturation, hemp seed oil is not generally recommended for frying or cooking. Analyses made by Mölleken (1998), however, have shown that cooking temperatures of about 170–250°C do not lead to an increase in harmful trans-fatty acids in hemp seed oil, as the results were similar to those measured in other edible oils, such as sunflower, safflower, soybean and walnut. This finding contradicts the generally accepted opinion that hemp seed oil only tolerates moderate heating (up to 150°C) for a short time without any change in its fatty acid content.

Various studies have dealt with the fatty acid composition of hemp seed oil. The subjects of these analyses were landraces or varieties of various origin, and the studies were conducted under different climatic and agrotechnical conditions. Analyses by Prjanisnikov (1930) show that the oil of several Central Russian landraces contained 13–14% LNA and only 6–7% LA. Callaway and Laakkonen (1996) and Callaway (2002) reported that the oil of variety FIN-314 (later named Finola) has a favourable composition, especially with respect to GLA. The oil of twenty varieties from six countries was analysed by Vogl et al. (2004) to compare their oil composition at three harvest dates. The results indicated that genotype had no significant influence on the fatty acid content.

Kralovánszky and Marthné Schill (1994) determined the fatty acid profiles of six Hungarian hemp varieties. A comparison of these results with those reported by Jáky (1946) indicates that the fatty acid profiles changed over the course of fifty years: the content of the essential fatty acids (EFAs) LA and LNA showed increases of 5% and 4%, respectively, while the relative amounts of oleic, stearic and palmitic acids decreased.

In 1996, selection for high seed oil content was started in the parental varieties Kompolti and Fibrimon 21-63. In the case of Kompolti the oil content increased by 3.0% on the basis of pressed oil and by 2.9% according to the data of extraction with hexane. In Fibrimon 21-63 these values were 1.4% and 0.9% over a period of six years (Bócsa et al., 2005). After two further years of selection, the oil of the two selected populations (Kolaj and Fibrol) and of the starting material (Kompolti and Fibrimon 21-63) was analysed to determine the effect of the increase in oil content on its composition.

The purpose of the present study was to compare the relative amounts of fatty acids in the seed oil of the initial populations (Kompolti and Fibrimon 21-63) with those of the selected populations (Kolaj and Fibrol) and to determine the degree and direction of the changes, with special respect to EFAs. The aim was to observe whether and how the FA ratios in hemp seed oil change as the result of selection for increased seed oil content.

Materials and methods

Varieties

a) Kolaj (improved Kompolti), breeding stock (dioecious hemp)
b) Kompolti kender (the parental variety from which Kolaj was selected.) A dioecious fibre hemp variety with very high fibre content, registered in 1955
c) Fibrol (improved Fibrimon 21-63). A monoecious hemp variety with high oil content, registered in 2006
d) Fibrimon 21-63 (the hybrid parental variety from which Fibrol was selected)

Experimental design

Half-sib families of the initial and the most recently selected populations were sown in alternate rows at a spacing of 70 × 70 cm.

Fatty acid analysis

Seed oil samples for the analysis of fatty acids were obtained by supercritical extraction with liquid CO_2. The samples were then hydrolysed with 1.5 ml methanol : NaOH 0.5 M solution and methylated with BF_3–methanol. Methylpalmitate (C17:0) was used as the internal standard.

Parameters of gas chromatographic analysis

Apparatus: Fisons GC 8060, AS 800; column: Omegawax 250, Supelco, Bellefonte, PA, USA, 30 m, i.d. 0.25 mm; injection temperature: 220°C; detection temperature 240°C (FID); carrier gas He; velocity 30 m/s; split ratio 1:50; temperature program: 50–220°C (4°C/min, 15 min isotherm at 220°C); injection volume: 1 µl.

Parameters of supercritical extraction:

Apparatus: Jasco 900, Japan; restrictor system: back pressure regulation; solvent: SC–CO_2 (purity 99.9995%, Linde, Hungary); pressure: 29 Mpa; duration of extractions: static 30 min, dynamic 30 min.

Determination of oil content by extraction

Fully mature plants were dried outdoors for a week after harvest. The seed yields of each plant were then threshed separately, cleaned and dried to the same moisture content (3%). Two g samples were taken from the yield of 12 plants from each population for the determination of oil content. The samples were crushed in a porcelain mortar. The oil was then extracted 10× on a Soxhlet apparatus using n-hexane as the extracting agent. After extraction, the oil content was determined gravimetrically.

Evaluation method

The data were evaluated and analysed using the two-sample t-test, as described by Sváb (1973).

Results and discussion

In both breeding populations the total oil content increased significantly and these values currently hardly differ (35.54% and 35.73%). However, a greater selection gain was achieved in Kolaj over the same time period (Tables 1 and 2). The interpretation of the superior gain in dioecious Kolaj is complex (Bócsa et al., 2005). There were at least three reasons for the smaller selection gain in Fibrol: 1.) Lack of seed samples of appropriate weight (50 g),

2.) Monoecism, which may cause about 20–26% of inbreeding (Horkay, 1986), 3.) Priority of selection for lower THC content.

The changes in the FA profiles of Kolaj and Fibrol were of opposite sign except for α-linolenic acid and the saturated acids (palmitic and stearic) (Fig. 1). While the stearic acid ratio did not change in Kolaj, that of palmitic acid decreased significantly. In contrast, there was a negligible change in the ratio of palmitic acid in Fibrol, while the ratio of stearic acid significantly increased.

According to human diet specialists, the presence of oleic acid is undesirable in edible oils (Kralovánszky and Marthné Schill, 1994), although a higher content in oils is favourable for frying. In the present study, the changes in the contents of oleic and linoleic acids in Fibrol and Kolaj were opposite and significant. Within each of Kolaj and Fibrol the changes in the oleic and linoleic acid ratios had the same sign and were of similar intensity, unlike the FA biosynthesis in sunflower (Frank, 1999). While the biosynthesis of oleic and linoleic acid shows a negative correlation in sunflower, allowing breeders to use the underlying genetic variation to select for stable high oleic or linoleic acid lines, the present results do not affirm these results for hemp.

Table 1

Differences in fatty acid profile between (A) control parental hybrid Fibrimon 21-63 and (B) selected Fibrol variety (n=12)

	Oil content		Palmitic		Stearic		Oleic		Linoleic		α-linolenic		γ-linolenic	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Mean (%)	32.38	35.54	5.72	5.70	2.36	2.85	15.69	14.18	58.09	56.85	15.93	15.08	1.40	2.03
Diff. of means (B–A)	3.16		–0.02		0.49		–1.51		–1.24		–0.85		0.63	
SE of mean diff.	1.13		0.65		0.08		0.63		0.38		0.61		0.26	
t-value	2.47		0.03		6.24		2.38		3.24		1.40		2.38	
Critical t-value	2.26		2.26		2.26		2.26		2.26		2.26		2.26	
$LSD_{0.05}$	2.89*		1.47		0.18*		1.43*		0.87*		1.37		0.60*	

Table 2

Differences in fatty acid profile between (A) control variety Kompolti and (B) selected Kolaj breeding material (n=12)

	Oil content		Palmitic		Stearic		Oleic		Linoleic		α-linolenic		γ-linolenic	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Mean (%)	29.86	35.73	6.33	4.57	2.56	2.56	10.93	12.58	58.73	61.16	19.73	16.75	1.12	0.94
Diff. of means (B–A)	5.87		–1.76		0		1.65		2.43		–2.98		0.18	
SE of mean diff.	1.64		0.67		0.11		0.46		0.60		0.54		0.11	
t-value	3.59		2.64		0.01		3.57		4.07		5.47		0.02	
Critical t-value	2.26		2.26		2.26		2.26		2.26		2.26		2.26	
$LSD_{0.05}$	3.70*		1.51*		0.26		1.04*		1.35*		1.23*		0.25	

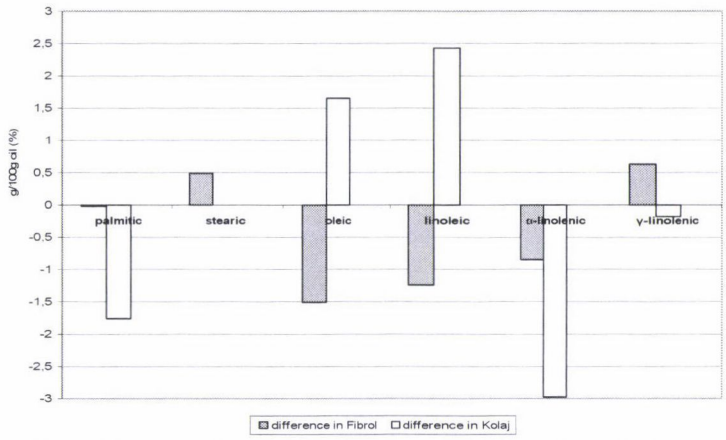

Fig. 1 Changes in the fatty acid profile after selection for high oil content

The content of α-linolenic acid decreased in both selected populations, while γ-linolenic acid significantly increased in Fibrol, but in Kolaj its content was less than in the initial breeding material.

The balance of linoleic and α-linolenic acid in the Kompolti variety is almost exactly 3:1 (58.7:19.7), which Erasmus claimed to be optimal for human nutrition (Deferne and Pate, 1996). Besides increasing the ratio between these two FAs, the increase in oil content was accompanied by an increase in the proportion of oleic acid and a decrease in that of γ-linolenic acid. Thus, the changes occurring in the fatty acid profile of Kolaj were nutritionally negative. The nutritional advantage of increasing the oil content in Fibrol was to increase the proportion of γ-linolenic acid.

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EFFECT OF FOLIAR FERTILIZER CAMPOFORT SPECIAL-Zn AND PLANT GROWTH REGULATOR RASTIM 30 DKV ON GROWTH, YIELD COMPONENTS AND PROTEIN CONTENT IN MUNG BEAN PLANTS

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The effect of the foliar fertilizer Campofort Special-Zn (CSZn) and the plant growth regulator Rastim 30 DKV (RM) on growth, yield parameters and seed protein content was studied in mung bean [*Vigna radiata* (L.) Wilczek] plants grown under greenhouse and field conditions. CSZn at a dose of 10 l per ha, and RM at doses of 3, 30 and 60 g per ha were applied alone or in combination (before flowering and 14 days after first application). The initiation of flowering and ripening processes and the chlorophyll content Chl ($a + b$) were evaluated. At harvest, total yield and yield components (number of pods per plant, seed number per pod, single pod mass, seed mass per pod), germination of seeds and seed protein content were determined. RM applied alone induced intensive flowering, increased the number of pods and yield components per plant, accelerated the ripening of the pods, increased the Chl content in the leaves and delayed senescence in treated plants. The mixture of RM with CSZn (60 g + 10 l per ha) and RM alone at a dose of 60 g per ha had a retarding and morphogenic effect on the growth of treated plants and also decreased the protein content and germination of the seeds. The best results for all studied parameters were achieved in the field at a dose of 30 g RM + 10 l CSZn and under greenhouse conditions at a dose of 3 g RM + 10 l CSZn.

Key words: foliar fertilizer Campofort Special-Zn, plant growth regulator Rastim 30 DKV, mung bean, yield, protein content, chlorophyll content, flowering, ripening

Introduction

Zinc is one of the essential micronutrients for plant systems and plays a fundamental role in protein and photosynthetic carbon metabolism (Prasad, 1995). In plants, zinc plays a key role as a structural constituent or regulatory co-factor in a wide range of enzymes in many important biochemical pathways (Brown et al., 1993). Zinc is absolutely essential for the healthy growth and optimum yield of all agricultural and horticultural crops (Brennan, 1991; Mortvedt and Gilkes, 1993; Rosolem and Sacramento, 2001). Physiological

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stress associated with zinc deficiency manifests itself in several different ways. These include: chlorosis of leaves, stunting, rosetting, dwarf and/or malformed leaves. All cases of zinc deficiency are accompanied by loss of yield and in many cases the quality of crop products such as grain or fruits is impaired as well. In fact, zinc deficiency is the most widespread of all micronutrient deficiency problems (Alloway, 2003). Zinc deficiency not only results in the restricted yield, growth and protein content of plants, but also affects its availability in human food, leading to various deficiency diseases (Lindsay, 1972; Graham and Rengel, 1993). Plants vary in their susceptibility to zinc deficiency, with beans and maize being among the most sensitive crops (Martens and Westermann, 1991). Zinc is more efficiently used by plants if applied before and during the leaf growth stage and before the appearance of the inflorescence (Brohi, 2000). Generally, microelements are added to macronutrient fertilizers during manufacture. However, microelements are also added to foliar fertilizers throughout the world as an effective, preventive and curative measure to compensate for their deficiency (Kassab, 2005; Thalooth et al., 2006). Foliar fertilizers containing different amounts of micronutrients were applied in many agricultural crops (Brohi, 2000; Khurana and Chatterjee, 2001; Pereira and Mello, 2002; Panayotov, 2006) and improved the yield and quality of treated crops. However, very little research has been carried out on the effect of leaf fertilizers combined with plant growth regulators on the yield and quality of plants (Pulkrábek et al., 2004).

The objective of this work was to study the influence of the foliar fertilizer Campofort Special-Zn and the plant growth regulator Rastim 30 DKV, alone and in combination, on the growth, yield components and protein content in mung bean plants.

Materials and methods

Plant material and soil

Mung bean plants were grown under semi-controlled greenhouse conditions in a soil culture using experimental pots (20 cm diameter) filled with sandy loam soil and farmyard manure, mixed in a 7:3 ratio. The pots, with one plant per pot, were arranged in the greenhouse according to a simple randomized block design with five plants per treatment in five repetitions. The field experiments were carried out on the experimental field of the Department of Plant Physiology, Comenius University in Bratislava (Slovakia) during the growing season in 2007. The mung bean plants were sown on 15 April in the greenhouse, and transplanted into the field by hand on 20 May. Plots were prepared using standard agronomic practices and each treatment had ten 1.5 m rows with a plant spacing of 20 × 30 cm. The size of the trial plots was 15 m^2 in five repetitions. Soil moisture was kept at adequate levels to prevent water deficit and wilting. The agrochemical characteristics of the soils is presented in Table 1. The soils were analysed at the Research Institute of Soil Science and Conservation in Bratislava. The soil reaction $pH_{(KCl)}$ was determined according to ISO 10390, humus according to ISO/FDIS 14235, hydrolysis of samples according to Kjeldahl, content of mineral elements (Mg, Ca, K) according to the methods FAAS ISO/DIS 14869:1998 and ISO/FDIS 11047:1997, phosphorus by the colorimetric method and total nitrogen according to STN ISO 11261.

Table 1

Agrochemical analyses of soil types used in greenhouse (I_G) and field (II_F) experiments. The analyses were carried out at the Research Institute of Soil Science and Conservation in Bratislava, Slovakia

Soil type	$pH_{(KCl)}$	Content of elements in soil (mg kg^{-1})*					Humus (%)
		N_{total}	P	Mg	Ca	K	
I_G	7.52	3 082	78.6	554.7	11 816	172.5	16.30
II_F	7.62	1 779	109.8	208.4	4 499	151.0	5.02

* Standard error did not exceed 10%

Treatment of plants

The foliar fertilizer Campofort Special-Zn (CSZn) (Agra Group Co., Střelské Hoštice, Czech Republic) and plant growth regulator Rastim 30 DKV (RM) (Istrochem, a. s., Bratislava, Slovakia) were obtained from the manufacturers. Campofort Special-Zn contains 180 g l^{-1} N, 43 g l^{-1} MgO, 34 g l^{-1} S and 12 g l^{-1} Zn in chelate form. Rastim 30 DKV is a wide-spectrum growth regulator. Its effective substance is 3-(benzyloxycarbonyl-methyl)-2-benzothiazolinone (30% v/v).

The mung bean plants were sprayed with CSZn and RM prior to flowering (at the stage of three trifoliate leaves) and again 14 days later at a dose of 10 l for CSZn and 3, 30 and 60 g for RM per hectare in a total volume of 1000 l water. The plants in the greenhouse were sprayed with the same doses at a rate of 10 cm^3 $plant^{-1}$. The day on which the first plants formed floral buds marked the onset of flowering. Foliar applications were done in the evening, when the relative humidity was high, as day temperatures exceed 30°C during June and July. Control plants were sprayed with distilled water only. In the greenhouse the mean minimum and maximum temperatures were 24.5 and 39.5 ± 2°C, relative humidity ranged from 35–60 ± 5%, maximum irradiance (PAR) was 1400 µmol m^{-2} s^{-1} and the photoperiod was 14–16 h, while in the field the temperature ranged from 18.5–33.2 ± 2°C during the growing season (May–September, 2007).

Evaluation of yield parameters

The representative samples were collected in five replicates for each treatment when the pods were fully ripe (black colour). Yield parameters (number of pods per plant, number of seeds per pod, single pod mass and seed mass per pod in g) were recorded from ten selected plants per variant under greenhouse conditions and from ten plants from the inner rows of each plot in the field, and seeds were taken for protein and germination analysis. The whole plot was harvested for the determination of seed and total yield per hectare.

Protein determination

Protein extraction was performed using 100 mg of mung bean seed powder and 1 ml of extraction buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 5% mercaptoethanol and 10% glycerol). The aqueous supernatants were obtained after 10 min extraction followed by 15 min centrifugation of the homogenates at 12,000 g. The soluble protein content in the aqueous supernatants was determined according to Bradford (1976).

Chlorophyll determination

Seven days after the second application, chlorophyll pigments *a* and *b* were extracted in 80% acetone from 3 × 1 g leaf tissues and determined as described by Lichtenthaler (1987) by measuring the absorbances at 665 and 649 nm using a UV-Vis spectrophotometer (Jenway Model 6400, Essex, England).

Germination test

Mung bean seeds harvested from treated and untreated plants were germinated in Petri dishes on two filter-paper discs. The Petri dishes contained 12 cm^3 of distilled water. The seeds were germinated for 72 h in an incubator at 25°C and 50% air humidity. Each treatment was repeated five times with one hundred seeds.

Statistical treatment of the data

The data were analysed in a one-way ANOVA test using SPSS 9.0 for Microsoft Windows. Comparisons between the mean values were made using an LSD test (least significant difference) at the 0.05 level.

Results

The combined application of CSZn and RM exhibited a positive effect on the growth and yield parameters of mung bean plants. The time to flowering was 2–3 days shorter in plants treated with RM alone or in combination than in the control plants (Table 2). Not only did RM enable the plants to retain the maximum number of flowers per plant, which matured into pods, but the flowering period of treated mung bean plants after the second application was very long and lasted to the end of August. The plants began flowering simultaneously and intensively in comparison to the controls. The flower shedding observed in plants grown in the greenhouse was the least in RM-treated plants.

The data presented in Table 2 show that the total content of Chl increased in the leaves of mung bean plants. Table 2 also shows that there was an insignificant increase of 2–6% (13.42–15.89 mg g^{-1} DM) in the chlorophyll content after the foliar application of CSZn, compared to the control (13.20–15.01 mg g^{-1} DM). The chlorophyll content was higher than in the control in both experiments when RM was applied alone, rising as the dose increased by 12–44% under greenhouse conditions and by 2–15% in the field. After treatment with RM + CSZn, the chlorophyll content increased by 4–26% over the control in both experiments (Table 2).

The foliar application of CSZn and RM alone or combined affected all the yield components, the increases being 11–45% in pod number per plant, 11–17% for individual pod mass, 7–23% for seed number per pod and 5–15% for seed mass per pod in the small-plot trials (Table 4). However, subjecting plants to temperature stress during vegetative growth in the greenhouse caused a greater reduction in all the yield parameters. The number of pods per plant was lower, and the seeds were smaller than those of plants under field conditions (Tables 3 and 4). The pods and seeds produced by plants treated with CSZn and RM at lower doses (3 or 30 g ha^{-1}) were for the most part indistinguishable from those of the control. However, plants treated with the highest dose of RM (60 g ha^{-1}) alone occasionally produced deformed terminal racemes, composed of pods greatly reduced in size. These abnormal pods remained green and contained either no seeds or rudimentary, non-viable ones. This negative effect on the pods was evident mainly in the pot experiments. The application of RM alone or in

combination with CSZn also had a positive effect on the ripening of the pods and the maturation of the crop both in the greenhouse and in the field. RM, alone or in combination with CSZn, accelerated pod maturation particularly at the first harvest (Table 2). In the pot experiments the percentage of the total pods per plant that had ripened by the first harvest was significantly higher when RM was applied alone (25–87.5%) or in the combination RM+CSZn (18.7–56%) than in the control (10.22%). In the small-plot trials, the percentage of ripened pods was 18.2–46% higher in the RM treatment and 17–48% higher in RM+CSZn than in the control (9.30%) (Table 2).

Tables 3 and 4 summarize the effects of CSZn and RM on the total yield per plant and per hectare. In the greenhouse RM had a significant effect on the yield components, resulting in increased seed mass and in total yields of 5.46–5.92 g per plant, an increase of 38–49% in comparison to the control yield of 3.96 g per plant (Table 3). Under the influence of CSZn alone, the yield increased by 5.03 g (27%) per plant. On the other hand, although RM alone exerted a significant effect on yield components, the presence of this growth regulator in combination with CSZn only increased the yield at RM doses of 3 or 30 g ha^{-1} (23–74%) (Table 3). Under field conditions, the effects of RM and CSZn alone or in combination were similar to those observed in the greenhouse, but the values achieved for all yield components were significantly higher. The combination of RM with CSZn increased seed yield at all RM doses from 34 to 64% over the control, with an average seed yield of 1381.1 kg ha^{-1} (Table 4). The total yield of seeds per plant (Table 3) or per hectare (Table 4) and the number of floral buds formed in a plant was the lowest in the combined treatment, CSZn and RM (10 l + 60 g ha^{-1}), for both experiments. The highest total seed yield per plant from all variants was achieved for the RM + CSZn combination in doses of 3 g + 10 l ha^{-1} in the greenhouse (Table 3) and 30 g + 10 l ha^{-1} under field conditions (Table 4).

Table 2

Changes in flowering, ripening and chlorophyll content of mung bean plants after foliar applications of Campofort Special-Zn (CSZn) or Rastim 30 DKV (RM) and their combinations under greenhouse (GC) and field conditions (FC)

		Control 0	CSZn 10 l	RM 3 g	RM 30 g	RM 60 g	CSZn+RM 10 l + 3 g	CSZn+RM 10 l + 30 g	CSZn+RM 10 l + 60 g
Flowering initiation^{+}	GC	9.20^{bc}	9.45^{c}	9.50^{c}	8.20^{b}	7.50^{a}	9.05^{bc}	9.50^{c}	8.30^{b}
	FC	12.50^{c}	12.90^{cd}	12.10^{c}	11.24^{bc}	10.50^{b}	12.65^{cd}	10.30^{b}	9.75^{a}
No. of ripe pods at 1st harvest (%)	GC	10.20^{a}	12.50^{ab}	25.00^{c}	71.85^{e}	87.50^{f}	18.75^{b}	25.00^{c}	56.25^{d}
	FC	9.30^{ab}	9.10^{a}	18.18^{b}	51.52^{d}	46.36^{c}	17.00^{b}	18.15^{b}	47.70^{c}
Chlorophyll content $(a + b)^{++}$	GC	15.01^{a}	15.89^{a}	16.90^{b}	19.93^{cd}	21.60^{e}	15.60^{a}	17.36^{bc}	18.90^{c}
	FC	13.20^{a}	13.42^{a}	13.55^{a}	14.20^{b}	15.25^{c}	13.25^{a}	13.98^{ab}	14.55^{bc}

First applications were done on 17.6.2007 in the greenhouse and 2.7.2007 under field conditions. First harvests of ripe pods were done on 25.7.2007 in the greenhouse and 10.8.2007 under field conditions; $^{+}$: after 1st application (days); $^{++}$ Chlorophyll content in the leaves in both experiments was determined 7 days after the second application, mg g^{-1} DM; Data followed by the same letters are not significantly different at P = 0.05 according to the LSD test

Table 3

Effect of foliarly applied Campofort Special-Zn (CSZn) or Rastim 30 DKV (RM) and their combinations on the yield parameters of mung bean plants grown under greenhouse conditions

Treatment	Dose ha^{-1}	Pod number $plant^{-1}$	Seed number pod^{-1}	Single pod mass g	Seed mass pod^{-1} g	Seed yield $plant^{-1}$ g
Control	0	8.83 ± 0.98^{ab}	9.42 ± 0.56^{ab}	0.59 ± 0.02^{ab}	0.43 ± 0.01^{a}	3.96 ± 0.42^{ab}
CSZn	10 l	9.33 ± 0.66^{b}	10.44 ± 0.38^{bc}	0.56 ± 0.02^{a}	0.48 ± 0.02^{ab}	5.03 ± 0.29^{bc}
RM	3 g	10.67 ± 0.84^{c}	10.87 ± 0.43^{bc}	0.67 ± 0.03^{bc}	0.50 ± 0.01^{b}	5.46 ± 0.46^{cd}
RM	30 g	10.17 ± 0.48^{c}	11.18 ± 0.82^{c}	0.73 ± 0.03^{c}	0.58 ± 0.02^{c}	5.92 ± 0.24^{d}
RM	60 g	8.81 ± 0.31^{ab}	8.93 ± 0.14^{a}	0.62 ± 0.03^{b}	0.47 ± 0.01^{ab}	3.57 ± 0.11^{a}
CSZn+RM	10 l + 3 g	11.50 ± 0.50^{d}	11.06 ± 0.68^{c}	0.74 ± 0.06^{c}	0.58 ± 0.04^{c}	6.92 ± 0.21^{e}
CSZn+RM	10 l + 30 g	8.56 ± 0.36^{a}	9.96 ± 0.27^{abc}	0.68 ± 0.04^{bc}	0.53 ± 0.03^{bc}	4.89 ± 0.25^{b}
CSZn+RM	10 l + 60 g	8.17 ± 0.60^{a}	9.12 ± 0.54^{b}	0.57 ± 0.03^{a}	0.45 ± 0.02^{a}	3.80 ± 0.39^{ab}

The preparations were applied twice: before flowering (17.6.2007) and two weeks after the first application (2.7.2007). Values represent means ± SE, n=5. Data followed by the same letters are not significantly different at P = 0.05 according to the LSD test

Table 4

Effect of foliarly applied Campofort Special-Zn (CSZn) or Rastim 30 DKV (RM) and their combinations on the yield parameters of mung bean plants grown under field conditions

Treatment	Dose ha^{-1}	Pod number $plant^{-1}$	Seed number pod^{-1}	Single pod mass g	Seed mass pod^{-1} g	Seed yield kg ha^{-1}
Control	0	14.60 ± 0.81^{a}	8.47 ± 0.38^{a}	0.74 ± 0.06^{a}	0.61 ± 0.02^{a}	1381.1 ± 42.27^{a}
CSZn	10 l	16.60 ± 0.51^{b}	9.11 ± 0.17^{ab}	0.72 ± 0.04^{a}	0.64 ± 0.02^{ab}	1767.5 ± 17.31^{b}
RM	3 g	18.00 ± 0.84^{bc}	10.01 ± 0.19^{cd}	0.84 ± 0.02^{b}	0.66 ± 0.01^{bcd}	1987.9 ± 47.57^{cd}
RM	30 g	19.80 ± 1.53^{bcd}	10.21 ± 0.26^{cd}	0.85 ± 0.03^{b}	0.69 ± 0.01^{cd}	2172.3 ± 56.68^{de}
RM	60 g	16.20 ± 0.73^{b}	9.66 ± 0.14^{bc}	0.82 ± 0.02^{b}	0.66 ± 0.02^{bcd}	1995.6 ± 56.53^{cd}
CSZn+RM	10 l + 3 g	20.20 ± 1.16^{cd}	9.69 ± 0.97^{bc}	0.87 ± 0.03^{b}	0.69 ± 0.01^{cd}	2027.2 ± 38.90^{cd}
CSZn+RM	10 l + 30 g	21.20 ± 0.86^{d}	10.42 ± 0.31^{d}	0.86 ± 0.03^{b}	0.70 ± 0.03^{d}	2274.3 ± 31.08^{f}
CSZn+RM	10 l + 60 g	17.40 ± 0.60^{b}	9.51 ± 0.29^{bc}	0.82 ± 0.03^{ab}	0.65 ± 0.01^{bc}	1851.3 ± 46.36^{bc}

The preparations were applied twice: before flowering (2.7.2007) and two weeks after the first application (16.7.2007). Values represent means ± SE, n=5. Data followed by the same letters are not significantly different at P = 0.05 according to the LSD test

The germination of seeds from treated plants was not negatively affected and the seeds had high germination rates (92–99%) in comparison to the control plants (95%). The seeds from plants treated with RM alone at a dose of 60 g per ha or in combination with CSZn had lower (12–15%) germination compared with the control.

Figure 1 shows the effects of CSZn and RM alone and in combination on the soluble protein content of treated seeds. Lower doses of RM and CSZn applied alone did not result in any significant changes in protein content, but RM at a dose of 60 g per ha, either alone or in combination, significantly decreased the levels of soluble proteins to 68–77 mg g^{-1} DM from the control value of 81 mg g^{-1} DM. The data presented in Figure 1 show that subjecting mung bean plants to temperature stress at flowering and pod formation in the greenhouse

significantly increased the soluble seed protein content in the control (90.4 mg g^{-1} DM) compared to that in the field trial plots (81.5 mg g^{-1} DM). The highest value of protein content in treated plants (89.13 mg g^{-1} DM) was recorded in plants grown in the greenhouse and treated with RM at a dose of 3 g per ha, while the lowest (63.3 mg g^{-1} DM) was recorded in plants sprayed with RM + CSZn in doses of 60 g + 10 l ha^{-1}. Under field conditions, the effect of the preparations was similar, but the highest protein content (83.1 mg g^{-1} DM) was determined for RM alone at a dose of 30 g per ha, which was not significantly different from the control (81.5 mg g^{-1} DM).

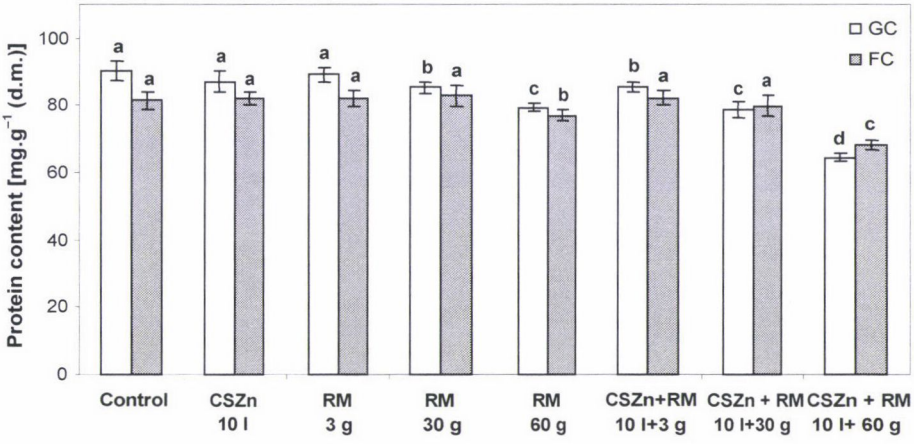

Fig. 1. Content of proteins (mg g^{-1} DM) in seeds of mung bean plants after foliar application of Campofort Special-Zn (CSZn) or Rastim 30 DKV (RM) and their combinations under greenhouse (GC) and field conditions (FC). Values represent means ± SE, n = 6. Data followed by the same letters are not significantly different at P = 0.05 according to the LSD test

Discussion

In agricultural practice, the application of leaf fertilizers containing micronutrients is very important as an intensification factor for plant productivity. Chelated forms of zinc are widely used and are usually applied early in the growing season. In a preliminary study (Henselová et al., 2007) zinc in chelate form, as ZnEDTA, was more readily translocated within the plant than that from other zinc sources. This was confirmed in the present work, when the leaf fertilizer Campofort Special-Zn (CSZn) was applied to mung bean plants. Besides CSZn, the influence of the plant regulator Rastim 30 DKV (RM), which has auxin-like growth activity, was also studied. The first application (before flowering) of RM alone or in combination with CSZn, affected the number of floral buds and the intensity of flowering. The second application improved the photosynthetic activity of the leaves and the transport of assimilates to the productive organs, the seeds. It is difficult to explain the interaction between RM

and CSZn. There only appears to be a synergistic effect when RM is present in the mixture at lower doses (3 g ha^{-1} in the greenhouse and 30 g ha^{-1} under field conditions). The stimulating effect of RM on mung bean yield seems to be partially related to the enhanced uptake of mineral nutrients from the soil and the better utilization of macro- and microelements. This corresponds with Brennan's (1991) report on the increased uptake of elements under the influence of humic acid substances and with the statement of Casenave de Sanfilippo et al. (1990) that the effect of humic acid substances is usually similar to that of plant growth regulators.

It is clear from the present results that mung bean plants appeared to be more sensitive to higher temperature and water stress during flowering and the pod formation stage, which resulted in the shedding of flowers and lower seed yield per plant in the greenhouse in comparison with field conditions. It seems that the foliar application of RM and CSZn increases the tolerance of mung bean plants to stress in the greenhouse, which was also confirmed by the results of Thalooth et al. (2006) with the foliar application of zinc, potassium and magnesium in mung bean plants. It can be concluded that spraying mung bean plants with RM and RM + CSZn enhances flower production, and accelerates and synchronizes the process of crop maturation. Similarly, the external application of benzyladenine and kinetin in wheat (Beckett and Van Staden, 1992) had a comparable effect.

The sensitivity of the tested mung bean plants to RM toxicity increased at the highest dose of 60 g ha^{-1}. Epinastic and formative effects were observed, with reduced growth area of the leaves. This negative effect of RM was observed mainly under temperature-stressed greenhouse conditions, while the symptoms for the same dose were insignificant in the field. It can be assumed that RM, as a synthetic auxinoid, probably reinforces the effect of endogenous IAA, resulting in an undesirable morphogenic effect on the leaves of treated mung bean plants. The foliar application of CSZn alone or in combination with RM, significantly increased all the yield characters compared with the control plants. These results are in full agreement with the findings of Brohi (2000) in wheat and Pulkrábek et al. (2004) in sugar beet after the application of Campofort fertilizers with different contents of microelements. Zinc application through CSZn alone was also effective with respect to yield. Similar positive effects of zinc foliar fertilizers on seed yields were reported by Jasper et al. (2000) in pea and Panayotov (2006) in sweet pepper. The present results suggested that the foliar application of CSZn and RM partially alleviated the adverse effects of water and temperature stress on photosynthesis and photosynthesis-related parameters, and on yield and yield components, by mitigating the nutrient demands of stressed plants. This is in agreement with Kumar et al. (2001) and Ved et al. (2002), who stated that the foliar application of growth regulators and zinc enhanced photosynthesis and the early growth of plants, and improved nitrogen fixation, grain protein and yields. A correlation was found in the present work between the number of pods, the seed mass per

pod or plant and the yield per plant or per ha, which corresponds with the results of Takahashi et al. (1994) in mung bean. The foliar application of zinc in the form of CSZn fertilizer also affects plant growth and mung bean production. Similarly, Kassab (2005) indicated that the foliar application of Zn, Mg, Mn and Fe significantly increased the growth parameters, yield and yield components of this crop. In the present study, RM delayed the senescence of leaves under both trial conditions, similar to cytokinins in the leaves of bean plants (Metwally et al., 1997). Rastim treatment resulted in significantly higher Chl contents as compared to the control, with a higher Chl content in plants grown under greenhouse conditions. RM delayed senescence in wheat segments (Klíčová et al., 1994) and also increased the content of endogenous phytohormones in tomato plants (Henselová et al., 2001). Therefore, it is likely that the stimulation of Chl synthesis and the earlier ripening of pods in RM-treated mung bean plants is mediated through an effect on endogenous cytokinins and the increased production of ethylene. It is likely that temperature stress induced earlier senescence in the greenhouse than under field conditions through the degradation of chlorophyll and proteins.

The treatment of mung bean plants with RM or CSZn alone had no effect on the protein content in the seeds. The decrease in protein content in the combined treatment (CSZn + RM in doses of 10 l + 60 g) was possibly due to a decrease in the protein metabolism, as ascertained in *Phaseolus vulgaris* under the influence of Zn treatments by Chaoui et al. (1997). The significantly higher protein content recorded in seeds from the greenhouse as compared to seeds from field trials can be attributed not only to the different growing conditions, but also to the higher seed yield per plant in the field than under greenhouse conditions. The decrease in the germination of seeds after the application of the highest dose of RM, alone or in combination with CSZn, could be due to the excessive accumulation of zinc and other micronutrients in the plants. This high concentration of elements may operate as a stress factor, causing physiological constraints and leading to decreased seed vigour, viability and plant growth, as described in the *Bacopa monniera* species (Ali et al., 1999).

The present investigation indicates that the combined application of the leaf fertilizer CSZn and the plant growth regulator RM could be beneficial to mung bean crops. The study suggests that RM in doses of 3 and 30 g ha^{-1} is equally well absorbed when applied on the leaves. When combined with CSZn at 10 l per ha, RM increased the yield parameters of mung bean plants. Both alone and in combination with CSZn RM was also able to protect plants from both temperature and water stress.

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DIHAPLOID INDUCTION ABILITY OF THREE CLONES OF *Solanum phureja* (2n = 2x = 24) IN INTERPLOIDY CROSS WITH *S. tuberosum* (2n = 4x = 48)

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Potato, *Solanum tuberosum* L. (2n = 4x = 48), is an autotetraploid species, the breeding of which at the tetraploid level is complicated by tetrasomic inheritance. Dihaploids (2n = 2x = 24) from the tetraploid cultivated potato have great potential for breeding and genetic studies. The common method deployed to obtain potato dihaploids is to make interspecific-interploidy (4x × 2x) crosses between a tetraploid seed parent and special clones from the diploid *S. phureja* as pollinator. Pollinators carrying a marker gene have been used, but unfortunately, these clones were very weak, with rare flowering and low male fertility under the given conditions. To find a suitable pollinator, three clones were selected from *S. phureja* based on flowering, pollen shed and male fertility and were crossed with five cultivated tetraploid potatoes to evaluate their dihaploid induction ability. A total of 1529 interploidy crosses were made, resulting in 1116 berries and 1456 seeds. The progeny were divided into two groups based on stem, flower and tuber colour: hybrids and non-hybrids (putative dihaploids). Chromosome counting in non-hybrid genotypes detected 39 dihaploids. The clone *phu 3* and cv. Picasso, with 12.1 and 10.7 dihaploids per 100 berries, respectively, were the best dihaploid inducer and seed parent for dihaploid production.

Key words: potato, *Solanum tuberosum*, *S. phureja*, dihaploid induction, interploidy crosses

Introduction

Despite having a potato production area of more than 180,000 ha, Iran has no intensive potato breeding programme and all the cultivars produced are of foreign origin, mainly introduced from Europe. Recently work has been started in the Department of Horticultural Sciences, University of Tabriz, to develop Iranian cultivars.

Due to the narrow genetic basis of commercial potatoes in general, and specifically under Iranian conditions where only a limited number of cultivars

are accessible, establishing a breeding programme is difficult, since such programmes normally benefit from diverse genetic resources. To create such a diverse genetic base, the use of wild tuber-bearing *Solanum* species is necessary. Among the wild potato species, 73% are diploid (Hawkes, 1992). The commercial potato is a tetrasomic tetraploid. This makes genetic studies and conventional breeding efforts difficult (Ercolano et al., 2004). Genetic studies and the capturing of germplasm from diverse wild diploid species can be simplified by reducing the potato ploidy to the diploid level by extracting dihaploids. The most common method used to obtain potato dihaploids is to make inter-specific, inter-ploidy crosses (4x × 2x) between tetraploid potato as seed parent and selected clones of diploid *S. phureja* as pollen source (pollinator) (Hanneman and Rudhe, 1978; Liu and Douches, 1993; Hutten et al., 1994).

One of the goals in this research was thus to extract dihaploids from tetraploid cultivars for further use in exploiting both diploid species and wild allotetraploid species via bridge species and ploidy manipulation (Panahandeh et al., 2008). Superior dihaploid inducer *S. phureja* clones carrying the embryo spot marker gene that facilitates the identification of dihaploids in interspecific crosses (Hermsen and Verdenius, 1973) were obtained from potato gene banks. Unfortunately, these clones did not work well under the climatic conditions in Tabriz and were very weak, with limited flowering, very low pollen shed and even in some cases complete male sterility.

This made it necessary to select clones with long duration of flowering, high pollen shed density, good male fertility and tuberization under long-day conditions from a *S. phureja* population to facilitate their clonal maintenance. The aim of this work was to assess the dihaploid induction ability of three selected clones in crosses with tetraploid commercial potato.

Materials and methods

Plant materials

Of the three clones of diploid *S. phureja* used in this study, which were selected for long duration of flowering, pollen fertility and tuberization, *phu 1* was the accession GLK 1497, while *phu 2* and *phu 3* were inter-accession hybrid clones selected from the cross of GLK 1497 × CGN 17669. Five cultivars of tetraploid potato, namely Bolesta, Cosmos, Kaizer, Mondial and Picasso, were used as seed parents. Scions were taken from all the male and female parents, grafted on to tomato rootstocks and planted under a simply constructed screen house.

Crossing, and assessing the progeny of interploidy crosses

Flower buds of female parents were emasculated approximately one day before opening, pollinated the next day with freshly harvested pollen of the male parent and tagged. Fruits were harvested at least four weeks later and kept in the laboratory until softening. Seeds were extracted by cutting the berries, treated with 1500 ppm gibberellin to break dormancy, and then planted in an open bed according to Wiersema (1982) to produce seedling tubers.

The seedlings were divided into two groups: hybrids and non-hybrids (putative dihaploids), based on haulm, flower and tuber colour at harvest time.

Cytological investigation

Male fertility and the 2n pollen frequency of the pollinators were assessed by staining the pollen samples with aceto-carmine glycerol. In each case, at least 300 pollen grains were counted. Chromosome counting was carried out for all the putative dihaploid groups and for some of the hybrid plants. Tubers were planted on perlite and after germination root samples were taken and treated with 0.29 g l^{-1} 8-hydroxyl-quinoline for 2.5–4 h, fixed in 3:1 ethanol : acetic acid for 24 h, transferred into 70% ethanol and kept in the refrigerator until needed. For analysis the roots were washed, hydrolysed with 1N HCl for 10 min and stained with aceto-iron hemathoxilin for 24 h. After washing for several minutes, they were squashed in a single drop of 45% acetic acid.

Results

Overall, 1529 interspecific pollinations were carried out, resulting in 1116 fruits and 1456 seeds. Planting these seeds produced a total of 413 tuberized plants, of which 235 genotypes were putative dihaploid plants (not exhibiting hybrid characters). Cytological analysis resulted in the identification of 39 dihaploids (2n = 2x = 24; Fig. 1) while the others, except for a few triploids, were tetraploids.

Effect of pollinator

The male fertility of the pollinators was high, but the 2n pollen frequency was very low and was not observed in *phu 1* (Table 1).

Data on the overall pollination, berry production, number of seeds, germinated seeds, tuber-producing plants and number of dihaploids, in total and per 100 berries, for each pollinator are presented in Table 2. There was little variation between the pollinators for fruit set and seeds per berry. Clones *phu 2* and *phu 3* had very similar results and had better fruit set and seeds per berry than *phu 1*. Considerable variation was found among the pollinators in the number of dihaploids per berry, and *phu 3* was the best pollinator, with 12.1 dihaploids per 100 berries.

Table 1

Frequency of stainable pollen and 2n pollen in the three pollinators. All values are given as percentages

Pollinator	Pollen stainability	2n pollen
phu 1	61.0	0.0
phu 2	87.0	0.2
phu 3	87.5	0.5

Table 2

Dihaploid induction ability of three clones of the diploid pollinator *S. phureja* in interploidy crosses with five tetraploid cultivated potatoes

Pollinator	No. of pollinations	Fruits (%)	Seeds (S/F)	Emerged plants	Tuberized plants	Dihaploids	†Ratio
phu 1	614	417 (67.9)	400 (0.9)	244	116	6	1.40
phu 2	582	443 (76.1)	578 (1.3)	372	175	2	0.45
phu 3	333	256 (76.8)	478 (1.8)	281	122	31	12.10

S/F: Seeds/fruit; †Ratio: Dihaploids/100 berries

Effect of seed parent

As shown in Table 3, variations existed among the seed parents for their response to fruit set, the number of seeds per berry and the number of dihaploids per 100 berries. Mondial, with 0.86 dihaploids per 100 berries and Picasso, with 10.7 dihaploids per 100 berries, had the lowest and highest dihaploid production capacity, respectively. Overall, both the pollinator and the seed parent were found to influence dihaploid extraction. There may also have been an interaction effect, but this was impossible to confirm statistically, as there were no replications in the experiment.

In addition to chromosome counting for 235 progeny grouped as putative dihaploids, chromosome counting was carried out for at least 16 hybrid progeny belonging to each pollinator. The results showed that most of them were triploids (2n = 3x = 36; Fig. 2). The frequencies of triploidy were 94, 80 and 67% for *phu 1*, *phu 2* and *phu 3*, respectively.

Table 3

Dihaploid producing ability of interspecific pollinations between five tetraploid cultivated potatoes and three *S. phureja* clones

Seed parent	No. of pollinations	Fruits (%)	Seeds (S/F)	Emerged plants	Tuberized plants	Dihaploids	†Ratio
Bolesta	153	132(86.2)	176(1.3)	130	54	1	2.20
Cosmos	457	452(98.9)	429(0.94)	259	83	1	1.25
Kaizer	172	101(58.7)	126(1.24)	75	41	2	1.98
Mondial	230	116(50.4)	159(1.3)	68	55	1	0.86
Picasso	517	315(60.9)	566(1.79)	365	180	34	10.7

S/F: Seeds/fruit; †Ratio: Dihaploids/100 berries

Fig. 1. Metaphase spread of dihaploid (2n = 2x = 24) extracted from cv. Kaizer

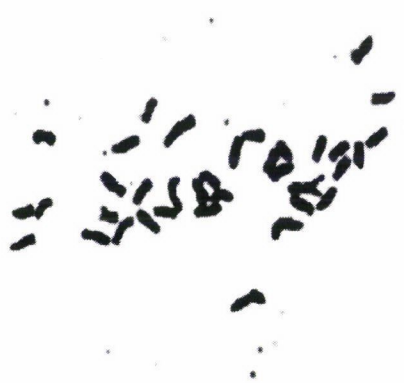

Fig. 2. Metaphase spread of triploid hybrid from Cosmos × *phu 2*

Discussion

In contrast to the finding of other authors (Liu and Douches, 1993; Kotch and Peloquin, 1987), high fruit set was observed, which was not expected from interploidy crosses. Taking into account that this experiment was carried out under uncontrolled conditions, these results may be related to the high male fertility of the pollinators (61, 87 and 87.5% for *phu 1*, *phu 2* and *phu 3*, respectively). Also, grafting to tomato rootstocks may have encouraged fruit retention in the seed parents, since this is comparable to the 76% fruit set reported by Hutten et al. (1994) using seed parents grafted on to tomato rootstocks.

The number of dihaploids per 100 berries for the three pollinators averaged 4.65 and was 12.1 for the best one. This was not as efficient as some superior dihaploid inducers (Hutten et al., 1994), but was comparable to the results published in some previous reports (Kotch and Peloquin, 1987; Dolnicar and Buhanich, 2000). However, considering that the clones here were selected based on male fertility and on the profusion and duration of flowering, the results of dihaploid production were significant. The classification of the progeny as hybrids or putative dihaploids was carried out at the tuber harvesting stage, because the pollinators did not carry the embryo spot marker gene used by most authors for selecting hybrids at the seed germination stage (Hutten et al., 1994; Caligari et al., 1988).

Picasso yielded more dihaploids than the other cultivars of *S. tuberosum*. This may be related to maternal effects on dihaploid frequency, as shown previously by Peloquin et al. (1996).

Some authors have reported the occurrence of aneuploidy in potato dihaploids extracted from interploidy crosses (Clulow et al., 1991; Samitsu and Hosaka, 2002), but all the dihaploids produced in this experiment were euploid ($2n = 2x = 24$).

The frequency of triploids in crosses between cultivated tetraploid and diploid potatoes was reported to be very low, which was first attributed to the triploid block and later explained by the endosperm balance number (EBN) hypothesis (for review, see Carputo et al., 1999; 2003). In the present experiment, however, most of hybrids were triploid rather than tetraploid. This does not question the EBN theory, but is related to the 2n pollen frequency, since there were very few 2n pollen grains in the pollinators. Nevertheless, in *phu 1*, where no 2n pollen was detected, 6% of the hybrid progeny were tetraploid, and in *phu 2* and *phu 3*, with 2n pollen frequencies of 0.2 and 0.5%, 20 and 33%, respectively, of the hybrid progeny were tetraploid. This demonstrates that even very rare 2n pollen is more likely to produce tetraploid seeds and confirms the power of EBN as a screening criterion in selecting 2n pollen for establishing a balanced ratio between parental EBN in the endosperm. Furthermore, as pointed out by Jackson et al. (1978), if the pollinators used in 4x × 2x crosses were strictly selected on the basis of dihaploid induction ability, the formation of triploids in progeny would be low, but unselected material increases triploidy, as observed in the present experiment.

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MOLECULAR FARMING, USING THE CEREAL ENDOSPERM AS BIOREACTOR

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Seed is an ideal protein production platform because it is the storage organ of the plant and offers appropriate storage compartments for the deposition of foreign proteins. To achieve high foreign protein expression level in the endosperm tissue, the transformation cassette carried the tissue-specific promoter of the wheat high-molecular-weight glutenin subunit protein 1Bx17, fused to the first intron of rice actin promoter. Transformation protocols were established and optimized in the laboratory for cereals such as rice, barley and wheat using direct DNA delivery and the *Agrobacterium tumefaciens*-mediated transformation system. Both immature (barley) and mature (rice) embryos, and immature inflorescences (wheat) were used as sources of explants. Subunit edible vaccines were produced to introduce the LTB, CTB and fused LTB-PEDV genes into the rice genome. The PEDV gene was also integrated into the barley genome. A project has recently been started to produce a rabbit-derived enzyme in transgenic wheat endosperm to be used by the pharmaceutical industry.

Key words: cereal transformation, edible vaccine, biofermentor, molecular farming

Introduction

Plants have been used by mankind for thousands of years, not only for food and feed, but also as raw materials and medicines. Apart from various plants organs, extracts also served to cure diseases. The systematic investigation of the therapeutic molecules allows these compounds to be used as a natural option in new drug production approaches. To overcome problems arising from inconsistent product quality and environmental effects, DNA technology has been introduced for the possible utilization of plant expression systems. One major achievement was the establishment of efficient plant transformation procedures both for dicots, based on either the nuclear or the plastid genome (Maliga, 2004), and for monocots (Vasil et al., 1992; Jones, 2005). Beyond the

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modification of agronomical traits, and the study of many aspects of gene function or of changes in the functional quality of plant products, the transgenic approach can also be used for the production of recombinant pharmaceutical proteins (Ma et al., 2003; Streatfield, 2007). Plants have become a convenient, safe and cheap alternative for foreign protein expression, replacing microbial or mammalian cell culture methods. Transgenic plant systems offer several advantages, including low energy input, easy control of production scale, and low risk of contamination by human and animal pathogens. The production of plants producing recombinant proteins or chemicals is known as "molecular farming" (Basaran and Rodriguez-Cerezo, 2008).

This technique has the potential to produce molecules in very large quantities for diagnostics, health care, and for the chemical and pharmaceutical industry. Depending on the promoter used in the transformation cassette to drive the transcription of the gene of interest, the recombinant proteins are expressed either constitutively or only in special organs. The use of tissue-specific, strong promoters has the advantage of enhancing the recombinant protein yield in transgenic crops (Twyman et al., 2003). Seed can be one of the most convenient protein production platforms, because it has several subcellular storage compartments for the deposition of the new proteins. Another advantage is the relatively high stability of the recombinant proteins, regardless of the storage temperature. In the case of vaccines or recombinant antibodies deposited in edible parts of the crops, no further processing or purification is required before utilization, providing a cold-chain- and needle-free vaccination process (Nochi et al., 2007)

An overview is given in this article of the efforts carried out at Eötvös Loránd University, in cooperation with national and international laboratories, to use the gene technology approach for producing cereal crops with novel properties for new applications.

Materials and methods

Plasmid constructions

The rice *act*1 first intron was amplified by PCR from rice genomic DNA, using the appropriate primers published earlier (Oszvald et al., 2008b), and fused with the 1Bx17 HMW-GS promoter in a transformation cassette designated as pTLZ (Oszvald et al., 2003). The resulting construct was identified as pTSI.

Rice transformation

Rice transformation was carried out as published by Cho et al. (2004). The embryos were separated from the endosperm after 7 days and were further cultured on N6 culture medium for callus production. After 3–4 weeks of cultivation the callus was bombarded using a "Genebooster". Transgenic callus was selected on medium containing 50 mg dm^{-3} of hygromycin B. Rapidly growing resistant tissues were moved to MS regeneration medium. Plantlets with 5–6 cm shoots and well-developed roots were transferred to soil and grown in a greenhouse.

Barley transformation and tissue culture

Immature barley embryos 1–2 mm in length were isolated from surface-sterilized caryopses. The embryos were placed on a Petri dish containing BCI medium and 0.4 ml *Agrobacterium* suspension (OD_{600}=2) was loaded on the top of them. The suspension was supplemented with 0.015% Silwet L-77. The embryos were transferred after draining onto new BCI medium without antibiotics for co-cultivation for 3 days. On the fourth day, the embryos were placed onto BCI medium supplemented with 75 mg/l hygromycin and 150 mg/l timentin for selection and callus induction. After the callus induction phase, the explants were placed onto DBC medium supplemented with 75 mg/l hygromycin and 150 mg/l timentin for 2–6 weeks, until greening was visible.

PCR analysis

The putative transgenic rice plants were screened for the selection marker gene and the transgene by PCR. Total genomic DNA was isolated from leaf, root and endosperm tissues using the Qiagen Plant DNeasy kit. PCR analysis for the genes was carried out using the appropriate primer pairs, specific for the given gene (either selection marker genes or a particular gene of interest). PCR products were analysed on 1% (w/v) agarose gel.

Immunoblot detection of CTB protein in transformed rice seeds

Total soluble proteins (TSP) were extracted from the mature seeds of transgenic rice plants. Around 100 mg of rice powder was mixed with 500 µl of buffer (200 mM Tris-HCl pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride and 0.01% Tween-20) and the homogenate was centrifuged for 15 min at 13,000 g at 4°C. The supernatant containing TSP was then subjected to further analysis. The protein was fractionated by 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond C membranes (Promega) in transfer buffer using a dry-blot apparatus (Bio-Rad, Hercules, CA). The membranes were incubated after masking with a 1:5,000 dilution of the appropriate antibody, developed in rabbit following the known protocol. Coloured bands were developed using the BCIP/NBT method in TMN buffer.

Quantification of CTB protein level in transgenic rice seeds

The expression of the edible vaccine protein level in the transgenic rice plants was determined by enzyme-linked immunosorbent assay (ELISA) following the method published by Oszvald et al. (2008b). Total soluble protein samples from the transgenic and wild-type plants were coated at 100 µl per well onto 96-well microtiter plates (Dynatech Laboratories, Burlington, MA) together with purified heterologously expressed proteins, and the plates were incubated overnight at 4°C. The plates were washed three times with PBST washing buffer and the background was blocked via incubation in 3% (w/v) bovine serum albumin (BSA). The plates were then incubated for 2 h in TBST containing the appropriate antibody in 1:5,000 dilution. After washing, the plates were incubated in a 1:7,000 dilution of anti-rabbit IgG conjugated with buffer containing alkaline phosphatase (Sigma A-2556) for 2 h at 37°C. After washing with PBST buffer the plates were developed by the addition of TMB substrates (PharMingen 2606 and 2607KC, Fallbrook, CA) at room temperature in darkness. The optical density was measured at 405 nm wavelength in an ELISA reader (Packard Instrument MRA-006, Meriden, CT). The edible vaccine protein expression level in the plant samples was quantified by comparison with known quantities of bacterial protein complex. All measurements were performed in triplicate, and analysis of variance was carried out using the statistical program of Excel (Microsoft Corp., USA).

G_{M1}-binding assay

G_{M1}-ELISA was conducted in an effort to determine the affinity of G_{M1}-ganglioside receptors for the plant-derived edible vaccine proteins following the method published earlier by Oszvald et al. (2008b).

Results and discussion

Research on the wheat endosperm-specific promoter

If seeds are taken as the target of the foreign proteins expressed in transgenic cereals there are several options for deposition. Tissue-specific promoters are available for either embryo (Cuming and Lane, 1979), aleurone (Kalla et al., 1994) or endosperm. Because the endosperm is much larger than the first two, endosperm tissue is the main target for recombinant protein expression. Promoters driving either the transcription of the genes for starch biosynthesis (Rasmussen and Donaldson, 2006) or the storage proteins can be used, but the promoters most frequently utilized in transformation cassettes are the storage protein genes deposited in the protein body. Both wheat prolamin and rice glutelin gene promoters are applied.

In promoter development experiments, the wheat high-molecular-weight (HMW) glutenin subunit promoter was used to construct an expression cassette based on an endosperm-specific promoter useful for high level recombinant protein expression in cereal "bioreactors". HMW glutenin subunit (GS) promoters are currently the most powerful endosperm-specific promoters. Bx type HMW glutenin subunits (encoded by the *Glu-B1-1* gene) are expressed at the highest level amongst the HMW-GS proteins (Juhasz et al., 2003). Storage protein genes do not contain introns. The gene expression level in plants has been proved to be increased by the addition of an intron to the promoter region of a heterologous gene (Vibok et al., 1999). Studies suggest that introns may improve the efficiency of mRNA processing. In plants, particularly in monocots, introns have been shown to contribute to the enhancement of gene expression in the case of constitutive promoters. McElroy et al. (1991) showed that the rice *act*1 first intron transiently increased the GUS enzyme expression level.

As reported earlier (Oszvald et al., 2008a), a chimeric promoter was assembled using the 5' UTR (1900 bp) of the gene coding for the 1Bx17 HMW glutenin subunit protein, responsible for tissue-specific expression and the first intron (456 bp) of the rice actin (*act*1) gene. The sequence around the initial translation codon was optimised. The effect of the intron and promoter regulatory sequences on the expression of the *uid*A gene was studied using different lengths of the 1Bx17 HMW-GS promoter. The functions of promoter elements, promoter lengths and actin first intron were tested by transient expression assay in immature wheat endosperm and in transgenic rice plants. The results showed that the insertion of the rice *act*1 first intron increased GUS expression by four times in transient assay.

Apart from the effect of the first intron of rice actin gene on the strength of the tissue-specific promoter, the length of the 1Bx17 HMW glutenin subunit promoter was also studied. Deletion of the far upstream region of the promoter proved to have no significant effect on the expression level; however, a substantial drop was observed when the length of the 5' UTR was reduced to

237 nucleotides. Previous studies on endosperm-specific promoters identified a primary enhancer sequence, the major regulatory element in HMW glutenin subunit genes. This motif starts 13 nucleotides upstream from the CAATTG sequence, cut by the *Mfe*I enzyme. To test whether the promoter (pTSIM) containing less than the complete enhancer fragment was sufficient for the tissue-specific driving of *uid*A gene expression, the pTSIM-GUS cassette was used to produce stable transgenic rice plants. Deletion of not only the N and E boxes but also the Cereal box and partial sequences of the HMW enhancer did not appear to affect the specificity of the 1Bx17 HMW-GS promoter in transgenic rice. The 1Bx17/*act*1 chimeric promoter showed strict tissue-specificity, being expressed only in the endosperm with no expression in any other tissues.

Research on cereal transformation

The genetic transformation of cereals has been accomplished in the last twenty years through various methods. Biolistics and agro-infiltration have become the most widely used techniques, because the best transformation efficiency can be reached through these approaches. Various type of explants such as mature and immature embryos, or immature inflorescences, can be used for biolistic transformation. Due to the shortage of space in the greenhouse and the time required for immature scutella production and preparation, the option of establishing a method based on mature seed was studied. A successful and efficient method was established to produce transgenic rice plants in the laboratory (Oszvald et al., 2007a). Apart from rice, wheat transformation was also achieved in two collaborating laboratories using the micro-projectile bombardment method (Sági et al., 2008). After intensive studies and the improvement of the tissue culture and regeneration approach (Tamás et al., 2004) a reliable procedure was established (Tamás et al., 2009). The inflorescence-based wheat transformation method also works successfully and can be used to produce transgenic wheat for molecular farming purposes (Jenes et al., unpublished results).

Since Tingay et al. (1997) reported the first *Agrobacterium*-mediated transformation of barley, the method has been set up in many laboratories, and many papers have suggested improvements, particularly in the efficiency of the protocol (Shrawat et al., 2007). A properly working procedure was also established by Eva et al. (2008) based on the variety Golden Promise, using minor modifications to the protocol published by Harwood et al. (2009). A transformation efficiency of 5% was achieved, which is comparable to the 6.7% reached by Shrawat et al. (2007). One reason for the successful transformation could be the use of Silwet-L77 in the inoculation medium and the tissue culture medium optimized by Harwood (personal communication). Silwet L-77 is a strong surfactant, which helps *Agrobacterium* cells to enter the plant tissue. The pre-regeneration medium used in the current work also contained 5 μM Cu^{2+}, which greatly affected the plant regeneration process (Purnhauser, 1991).

Research on edible vaccine production

Plant-based vaccines appear to provide promising examples of a new strategy that combines innovations in medical science and plant biology to generate affordable pharmaceutical products. Several plants have already been studied for their potential use in edible vaccine production and some have reached the phase of clinical trials (Streatfield, 2006). Based on previous intensive studies it can be concluded that vaccine production in plants is a promising cheap alternative in the fight against epidemic diseases, because modified plants could be grown locally, and the administration of the vaccine is safe and easy.

Efforts to generate recombinant proteins in plants have been focused on dicotyledonous plants, mainly including potato, tobacco and alfalfa. These plants, however, have some obvious disadvantages. Green leaf tissues harbour phenolic compounds, as well as a host of other potentially toxic compounds. They are also generally unpalatable, and any useful proteins generated must be extracted and purified before consumption. Cereal grains have a substantial advantage over green tissues. The yields of recombinant proteins tend to be much higher. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized in protein bodies.

Three GM rice varieties producing epitope vaccine have been produced (Oszvald et al. 2007b; 2007c; 2008b), two of which carry a synthetic sequence, with a sequence modification based on plant-optimised codon usage, coding for the non-toxic B subunit of either the *Escherichia coli* heat-labile enterotoxin (LT) or the *Vibrio cholera* cholera toxin (CT). These sequences were fused to a translation signal (the Kozak sequence) on the 5' end and the ER retention signal, SEKDEL, was added to the C terminus of the protein. The synthetic sequences were inserted into the pTSI plant transformation cassette (Oszvald et al., 2008a) under the control of the chimeric rice actin and wheat Bx17 HMW glutenin promoter. More than twenty hygromycin-b resistant rice lines were regenerated from both transformation events and subjected to further analysis at both the nucleotide and protein levels. The results of RT-PCR revealed that the genes of interest were transcribed neither in the leaves nor in root tissues, while Western blot analysis showed a reasonable level of recombinant proteins in the endosperm tissue.

Functional LTB and CTB proteins were synthesized as monomers, which were subsequently assembled into pentameric structures and deposited in protein bodies. The pentamer formation was confirmed by GM_1-ganglioside binding assay, which is located on the surface of eukaryotic cells. The expression level of both B subunits, measured by quantitative ELISA, varied between 0.5 and 2.7% of the total soluble protein (TSP), which represents about 1.3 mg/g seed recombinant protein. According to Tacket et al. (2004) this level of protein expression is sufficient to generate a sizeable amount of antigen after the consumption of a few milligrams of seeds, and the transgenic rice lines can be used for the production of rice seed-based edible vaccines.

Mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses in both systemic and mucosal compartments. However, some epitopes cannot be delivered directly to mucous membranes, but require a carrier protein to elicit a mucosal response. The Porcine epidemic diarrhoea virus (PEDV) has been identified as a member of the Coronaviridae family of viruses. It has been demonstrated to induce acute enteritis in pigs. The neutralizing epitope of PEDV was identified on the basis of sequence information for the same epitope of the transmissible gastroenteritis virus (TGEV). Efforts were made to develop a plant-based vaccine, produced in either rice or barley endosperm, via the coupling of a synthetic PEDV epitope to a synthetic LTB sequence. Both components of the fusion proteins were detected in the endosperm tissue via Western blot analysis. The fusion protein was demonstrated to assemble into pentamers, as evidenced by its ability to bind to GM_1-gangliosides. An appropriate level of expression (1.9% of TSP) was measured in the transgenic rice endosperm, allowing the successful development of a fusion-type edible vaccine.

Genetically modified cereal plant lines can be integrated into conventional breeding and, after intensive examination and evaluation processes to confirm the safety of the GM-related new lines, the new varieties should contribute to the development of safe edible vaccine production (Bedő, 2002).

Research on natural biofermentors producing enzymes of industrial interest

In recent decades the preparation of enantiomerically pure compounds has received exceptional attention. The synthesis of optically active materials represents a challenge both to academic and industrial chemists. The recognition of the fact that chirality plays a crucial role in nature encouraged tremendous efforts in enantioselective synthesis. According to the results of robust experiments it has been confirmed that enantiopurity is related to biological properties. Opposite enantiomers act differently within an organism and may display various activities. Some of these differences could be life-threatening, as they may result in teratogenic properties. The racemic drug thalidomide caused severe birth defects when taken by pregnant women.

There are a number of ways to produce enantiomerically pure compounds, but they are either very expensive (metal catalysis) or put enormous pressure on the environment (diastereomeric recrystallization). Asymmetric synthesis is generally used in natural product synthesis and in the industrial production of pharmaceuticals, flavours, fragrances, pesticides, etc. Among the variety of methods available for the synthesis of enantiomerically pure compounds, the application of enzymes has become accepted as a routine procedure. Although the concept of enzyme application to asymmetric synthesis has been long recognized, it is only recently that these catalysts have attracted attention.

The enzymes used for this reaction may show a high degree of substrate specificity in catalysing the transformation of their natural substrate. They often accept a wide range of structurally related compounds; for example, carboxyl esterase enzymes are able to work on phosphate ester molecules. Enzymes derived from animals cannot be used for drug production, as they are prohibited in the pharmaceutical industry, because of the possible contamination by animal-related viruses or prions. These enzymes, however, can be produced in transgenic plants and utilised by the pharmaceutical industry after extraction and purification, because the risk of contamination is negligible. Studies on rabbit liver esterase enzymes proved that only one isoenzyme has the ability to react with the appropriate molecule to produce an ester molecule with the desired chirality.

The protein was sequenced and the correct sequence was pooled out from a cDNA library for cloning into the pTSI plant transformation cassette and introduction into the wheat genome using the micro-projectile bombardment method. Several hundreds of immature inflorescence-derived calli were bombarded and more than a dozen plants were regenerated after callus selection. Samples were taken from the leaves and the integration of the sequence of the gene of interest was confirmed by PCR (Jenes, unpublished results). The next generation is being grown in a controlled environment for further studies.

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EFFECT OF DIFFERENT TILLAGE SYSTEMS ON THE YIELD AND YIELD COMPONENTS OF SOYBEAN [*Glycine max* (L.) Merr.]

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Eight different tillage systems were compared in soybean production on one experimental field (chernozem) located in the Baranya region of Croatia over a 4-year period (2001/2002, 2002/2003, 2003/2004, 2004/2005). The dry conditions experienced in 2003 exacerbated the effects of NT and CWNS on the soybean yield. The most stable grain yield was obtained using CSNW and CSDW in all four experimental years. DH, CH and CWDS did not result in any significant reduction in crop yield compared to CT. There was no clear trend regarding the applied tillage systems and grain yield components. The greatest effects on soybean yield and yield components were due to climatic conditions. Different tillage systems had a significant effect on the soybean grain yield and yield components in the four experimental years. The largest differences in stem height were determined between CSNW and NT. The number of pods per plant, the hectolitre mass and the grain yield were significantly lower under NT than under the other tillage systems. The number of fertile nodes of soybean and the number of branches per plant in the experimental years had approximately the same values for all the tillage systems. To sum up, the results achieved with DH, CH, CSDW, CWDS and CSNW were on par with each other and slightly better than CT, and these systems could represent adequate replacements for conventional tillage. No tillage could not be considered as the most favourable for soybean growing.

Key words: reduced tillage, grain yield component, soybean, grain yield

Abbreviations: CT: conventional tillage; DH: disc harrowing (fine till); CH: Soil loosening (chisel plough); NT: no tillage; CSDW: conventional tillage for soybean (odd years) and disc harrowing for winter wheat; CWDS: conventional tillage for winter wheat (odd years) and disc harrowing for soybean (even years); CSNW: conventional tillage for soybean (odd years) and no tillage for winter wheat; CWNS: conventional tillage for winter wheat (odd years) and no tillage for soybean (even years); RT: reduced tillage; Y × TS: Year × tillage system.

Introduction

Soybean [*Glycine max* (L.) Merr.] plants grown with no tillage often appear to be smaller than those grown with conventional tillage, yet they produce a similar grain yield (Yusuf et al., 1999). Conventional tillage practices are one of the many emerging environmental agronomic and economic issues that are addressed in contemporary cropping systems (Stevenson et al., 1998), in line with the European Community's Agricultural Policy, which strongly encourages soil-conserving tillage practices in order to decrease soil loss (European Union, 2000). Soybean production in eastern Croatia is based on mouldboard ploughing (25, 30 cm) and standard seedbed preparation (disc harrowing, harrowing, seeding). No tillage production results in changes in soil physical properties (Jug et al., 2001), including an increase in soil organic matter content (Douglas and Goss, 1982), aggregate stability (Birkás et al., 2002) and macroporosity (Lal et al., 1990). The changes may be detrimental, neutral or beneficial for crop growth and yield, depending on the soil texture and structure (Dick and Van Doren, 1985) and on climatic factors such as rainfall or drought (Morrison et al., 2000; Birkás and Gyuricza, 2004; Sabo et al., 2007). In general, NT systems have greater effects on the crop when used on poor soil, rather than on well-structured soil (Kladivko et al., 1986). Differences in yield components and their development emphasize the complexity of plant compensation in response to management system and tillage system (Pederson and Lauer, 2004). Much of the environmental variation in the yield of soybean and other grain crops is associated with variation in pods and seeds per unit area. While there is a wealth of data in the literature describing the general process of pod and seed set, the mechanisms regulating the number of seeds per unit area, an important yield component in soybean, are not well understood. The number of pods or seeds that the crop community produces is determined during this period and this number determines, in large part, the final grain yield. Much of the variation in soybean yield and other grain crops is associated with changes in the number of pods and seeds per unit area (Egli, 2005).

The objective of this study was to determine the effect of eight different tillage systems on soybean grain yield components including grain yield, plant spacing per square metre in the vegetative stage, stem height, number of branches per plant, number of fertile nodes, number of pods per plant, 1000-grain mass and hectolitre mass.

Materials and methods

Field experiments were conducted at the Kneževo site in the Baranya region of north-eastern Croatia (45° 32' N and 18° 44' E, 90 m elevation). The study was conducted over a 4-year period (2001/2002, 2002/2003, 2003/2004 and 2004/2005) as a monofactorial trial with randomized plots divided into blocks with four replications and with a basic plot area of 900 m^2 (18 × 50 m), set up on chernozem, the dominant soil type of the Baranya region. The soil

parameters were as follows: $pH_{(H2O)}$ = 8.14; $pH_{(HCL)}$ = 7.58; 2.79% organic matter; 120.9 mg kg^{-1} P and 131.8 mg kg^{-1} K, determined by the Egner-Riehm Domingo method (Page, 1982), and 2.55% $CaCO_3$. The climatic conditions during the experiment are shown in Table 1. The study began in 2001/2002. The experiment was conducted on the same homogeneous field at the same location in each experimental year. Before the experiment, only conventional tillage was applied. In all three years the forecrop was winter wheat. Soybean cultivar Tisa was sown at a rate of 120 kg ha^{-1} on April 27, 2002, April 24, 2003, April 29, 2004 and May 3, 2005 in all the tillage systems. The soybean emerged on May 17, 2002, May 13, 2003, May 12, 2004 and May 17, 2005. Fertilization was uniform for all the tillage systems and all the experimental years (40 kg ha^{-1} N, 130 kg ha^{-1} P and 130 kg ha^{-1} K as basic dressing).

The following treatments were applied each year:

(1) Control: conventional tillage (CT) involving autumn ploughing (30 cm deep), disc harrowing (DH) (15 cm) and disc harrowing to a depth of 10 cm. A John Deer 750A grain drill was used for all the tillage systems at a depth of 5 cm.

(2) Autumn disc harrowing (DH) was applied (fine till) to a depth of 15 cm and 10 cm, followed by seeding.

(3) Autumn disc harrowing + soil loosening was performed with a chisel (CH) to a depth of 20–30 cm and disc harrowing to a depth of 15 cm, followed by seeding.

(4) No tillage was followed by direct seeding.

In the following treatments different tillage systems were applied each year:

(5) CT for soybean with autumn disc harrowing to depths of 15 cm and 10 cm for wheat, followed by seeding, in the subsequent year (CSDW).

(6) Conventional tillage for wheat and disc harrowing for soybean in the subsequent year (CWDS).

(7) Conventional tillage for soybean and no tillage for wheat in the subsequent year (CSNW).

(8) Conventional tillage for wheat and no tillage for soybean in the subsequent year (CWNS).

Herbicides were applied as follows: in the case of NT and CWNS treatments, the total herbicide Hercules (*Glyphosate*) was used twice: I) in August after the pre-crop winter wheat harvest, and II) in April, two weeks prior to soybean seeding. The post-sowing/pre-emergence herbicides applied a few days after the seeding dates were *Frontier 900 EC* (*Dimetenamid*) 1.4 l ha^{-1} + *Tor 70 WP* (*Metribuzin*) 0.7 kg ha^{-1} in 2002, *Sencor WP 70* (*Metribuzin*) 0.7 kg ha^{-1} in 2003 and 2004, and *Sencor WP 70* (*Metribuzin*) 0.6 kg ha^{-1} + *Dual 960 EC* (*Metolachlor*) 1.2 l ha^{-1} in 2005. The corrective herbicides applied were *Dynam 75 WG* (*Oxasulfuron*) 90 g ha^{-1} on 23 May 2002, and *Bastional* (*Halixifop-esteri*) 1.3 l ha^{-1} on 11 June 2003, 18 June 2004 and 15 June 2005.

The long-term monthly precipitation and air temperature means at Kneževo and the average total precipitation and temperature during the 2002, 2003, 2004 and 2005 growing seasons are presented in Table 1. The total precipitation during the growing season was greater than the 30-yr average of 372 mm in three out of four years, while in 2003 it was only 180 mm, which was far lower than the 30-yr average.

Plant sampling was done five times during the 2002, 2003, 2004 and 2005 soybean vegetation seasons: in the vegetative stage V3–4 and in the reproductive phases R1 (beginning of flowering), R2 (full flowering), R3–4 (early and late pod formation) and R8 (full maturity). Phenological observations were made using the Fehr and Caviness (1977) growth stage key. The following soybean quality parameters were determined: plant spacing per m^2 in V3, height of stem (cm) in R1; number of branches per plant in R2; number of fertile nodes in R3; number of pods per plant, 1000-grain mass (g), hectolitre mass (kg) and grain yield (t ha^{-1}) in R8. The influence of different tillage systems on the yield components of soybean was investigated by variance analysis and tested using the F-test (P=0.01**; P=0.05*).

Table 1
Total precipitation (mm) and temperature (°C) in winter (October to March) and during the growing season (April to September) at the Kneževo site in 2001/2002, 2002/2003, 2003/2004 and 2004/2005

Seasons	Precipitation (mm)					Temperature (° C)				
	2002	2003	2004	2005	30-yr mean	2002	2003	2004	2005	30-yr mean
Winter	169	222	332	384	265	6	6	5	5	5
April	65	9	119	54	49	11	11	12	12	11
May	141	33	77	55	58	19	20	15	17	17
June	36	20	114	88	88	22	25	20	20	20
July	97	61	41	168	68	24	23	22	21	21
August	74	23	52	155	54	22	25	22	20	21
September	60	34	43	82	55	16	16	16	18	16
Growing season	473	180	446	594	372	19	20	18	18	18

Results

Plant spacing per m^2 in V3-4 (vegetative stage)

The highest plant spacing per m^2 in V3–4 over the four years was observed in the first (2002) and fourth (2005) years and the lowest in the second experimental year (2003). On average, the highest values for plant spacing per m^2 were found for the control (CT) and in the CWDS treatment, and the lowest for no tillage, whereas the other tillage systems were approximately the same (Table 2). According to the F-test, the year effect was very significant (F=107.13**), with a variation coefficient of 152.2%. However, the different tillage systems had a significant influence on the plant spacing per m^2 in V3-4 (F= 5.27**) and the TS × Y interaction was significant (F=1.95*) (Table 2).

Height of stem in R1 (reproductive phase – beginning of flowering)

The lowest stem height was recorded for no tillage (80.8 cm), and the highest after conventional tillage for soybean with no tillage for wheat (91.8 cm). The other tillage systems applied did not result in a significant reduction in stem height compared to the control (Table 2). The analysis of variance and F-test showed that the tillage systems had a significant influence on the stem height (F=5.06**), with a variation coefficient of 13.6%. In the first experimental year (2002) the soybean stem height was significantly greater than in the other years (P<1%). According to the F-test, the year effect was very significant (F=36.87**).

Number of branches per plant in R2 (full flowering)

Over the 4-year average, the number of branches per plant of soybean was approximately the same in all the tillage systems. Analysis of variance and the F-test showed that the tillage system had a significant influence on the number of branches per plant (F=4.52**). The biggest difference in the number of branches per plant, on a 4-yr average, was observed in the first year (2002) and the lowest in the second year (2003) (Table 2). According to the F-test, the year effect was very significant (F=10.02**) (Table 2).

Table 2
Analysis of variance and mean for yield components of soybean as affected by reduced tillage at Kneževo from 2002 to 2005

Variable	Plant spacing/m^2 in V3–4	Stem height (cm) R1	No. of branches/ plant R2	No. of fertile nodes R3	No. of pods per plant	1000-grain mass (g)	Hectolitre mass (kg)	Grain yield (t ha^{-1}) R8
Year								
2002	58	91.8	1.8	11.6	33.0	184.7	70.3	3.38
2003	23	77.6	2.9	11.8	58.5	147.8	71.6	2.19
2004	43	89.3	2.6	10.7	43.1	155.5	73.5	2.95
2005	57	85.7	2.8	11.5	44.0	152.2	75.0	2.69
Average (Y)	45	86.1	2.5	11.4	44.7	160.1	72.6	2.80
Tillage system								
CT (control)	49	86.7	2.4	11.3	45.9	166.1	73.3	2.92
DH	46	88.3	2.3	11.4	48.5	150.2	72.2	2.78
CH	44	85.7	2.8	11.3	47.7	163.9	72.8	2.91
NT	36	80.8	2.4	11.1	39.5	157.2	71.7	2.17
CSDW	45	87.5	2.4	11.4	42.4	155.7	72.8	3.08
CWDS	49	84.5	2.8	11.6	46.5	165.3	72.4	2.83
CSNW	48	91.8	2.1	11.6	42.2	163.4	73.0	3.10
CWNS	44	83.4	2.8	11.6	44.4	158.7	72.9	2.63
Average (TS)	45	86.1	2.5	11.4	44.7	160.1	72.6	2.80
				F-values				
Tillage systems	5.27**	5.06**	4.52**	0.62^{NS}	3.00**	2.18**	4.12**	25.30**
Year	107.13**	36.87**	10.02**	6.22*	85.07**	39.97**	44.77**	151.97**
Y × TS	1.95*	1.14^{NS}	1.05 ^{NS}	2.37**	2.10**	1.35 ^{NS}	1.90*	3.38**

NS: not significant; *, ** Values significant at the P<0.05 and P<0.01 level of probability

Number of fertile nodes in R3 (early and late pod formation)

The tillage systems applied had no significant influence on the number of fertile nodes (F=0.62). According to the F-test, the year effect was significant (F=6.22*), with a variation coefficient of 10.3%, and the interaction TS × Y was very significant (F=2.37**) (Table 2).

Number of pods per plant

Over a 4-year average, the highest number of pods per plant was achieved with DH and CH, and the lowest with NT, whereas the other tillage systems did not result in a significant reduction compared to CT. According to the F-test, the influence of the tillage systems on number of pods per plant was very significant (F=3.00**; 22.8%), but even greater differences in the number of pods per plant were determined between the years (Table 2). The analysis of variance and F-test showed that year had a very significant influence on the number of pods per plant (F=85.07**), with a variation coefficient of 77.3%. The interaction TS × Y was very significant (F=2.10**) (Table 2).

Thousand-grain mass

Over a 4-yr average the 1000-grain mass was 160.1 g, the lowest value being obtained for disc harrowing (150.2 g) and the highest in the control and CWDS treatments (166.1 and 165.3 g, respectively). According to the F-test, the tillage systems had a very significant influence (F=2.18**). The greatest differences in 1000-grain mass were observed between the years. The differences were very significant (F=39.97**).

Hectolitre mass

According to the F-test, the influence of different tillage systems on the hectolitre mass was very significant (F=4.12**), as were the differences between the years (F=44.77**). The interaction TS × Y was also significant (F=1.90*) (Table 2).

Grain yield

The lowest grain yield of soybean was obtained with no tillage (2.17 t ha^{-1}) and the highest with conventional tillage for soybean after no tillage or disc harrowing for wheat (3.10 t ha^{-1} and 3.08 t ha^{-1}, respectively), whereas the other tillage systems resulted in approximately the same values. The grain yield in 2003 averaged 2.19 t ha^{-1}, which was lower than in the other years (Table 2). Variance analysis and the F-test showed that the year had a very significant influence on the grain yield (F=151.97**), as did the tillage systems (F=25.30**). The year × tillage system interaction was significant and could possibly explain why higher soybean yields were recorded in 2002 in all the tillage systems than in the other years in the present study (Table 2).

Discussion

In general, 2002, 2004 and 2005 were wetter than the long-term mean, while 2003 was drier than the mean during the growing season (Table 1). The moisture deficits that occurred from April through September 2003 reduced the yields of the soybean cultivar used in this study, which is in accordance with many authors who emphasized the impact of climatic conditions on the grain yield (Morrison et al., 2000; Sabo et al., 2007). According to Sabo et al. (2007) the increased drought stress in 2003 was probably responsible for the lower seed yields (Table 2), by exacerbating the negative effects of no tillage on the soybean yield. The impact of climate conditions, tillage systems and the interaction Y × TS on the plant spacing per m^2 in V3–4 was significant in 2003 for all the tillage systems (Table 2). In the present study the plant spacing per m^2 in V3-4, the stem height, 1000-grain mass and grain yield of soybean were significantly lower under no tillage than in the control and other tillage systems. There was also a significant decrease in the soybean yield when no tillage

soybean was alternated with conventional tillage to wheat in the experimental years. According to Buzzell and Buttery (1977) the soybean grain yield was in strong and positive correlation with stem height, number of branches per plant and 1000-grain mass. In the present work the greatest differences in the number of pods per plant and the 1000-grain mass were determined between the years. The number of pods per plant was greater in the DH, CH and CWDS treatments than in the control and the other tillage systems. On a 4-yr average the lowest number of pods per plant was attained with no tillage. The highest variation in the number of pods per plant was recorded in the second year (2003), but the 1000-grain mass and grain yield of soybean were lowest in this year in comparison to the other years (Table 2). The year had a significant impact on the number of pods per plant (F=85.07**), while the 1000-grain mass was affected by the tillage system (F=2.18**). The greatest differences in 1000-grain mass and hectolitre mass were determined between the years, with a significant difference in 2003. According to the F-test, there was a very significant impact of year (F=44.77**) and tillage systems in this study (F=4.12**). The highest grain yield was achieved in the first year (2002), in comparison to the other years. The year × tillage interaction was significant and could also explain why a higher soybean yield was recorded in 2002 for all tillage systems than in the other years (Table 2). Averaged over four years, the highest grain yield of soybean was attained by conventional tillage for soybean after no tillage for wheat (3.10 t ha^{-1}) and conventional tillage for soybean after disc harrowing for wheat (3.08 t ha^{-1}), whereas the other tillage systems resulted in approximately the same values (Table 2). In the present study, the tillage systems with conventional tillage for soybean (CT, CSDW, CSNW) or loosening with a chisel plough each year were more productive than the other tillage systems applied and resulted in good soybean quality, so they can be recommended. Continuous disc harrowing or chisel ploughing, or disc harrowing for soybean only did not result in significant yield reductions compared to the control and produced soybean quality slightly better than the control.

Conclusions

Reduced tillage was found to have a highly significant impact on the yield and yield components of soybean in all the experimental years over a 4-year period. Yields decreased in the various tillage systems in the following order: CSNW>CSDW>CT>CH>CWDS>DH>CWNS>NT. Disc harrowing, chisel ploughing and disking for soybean after conventional tillage for wheat produced equal soybean quality and were slightly better than the control, so these systems could be recommended as satisfactory replacements for conventional tillage. No tillage, either for both crops or only for soybean, could not be considered as favourable for soybean production.

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APPEARANCE OF MICROFUNGI IN MAIZE STALKS DUE TO INJURIES CAUSED BY THE EUROPEAN CORN BORER (*Ostrinia nubilalis* HBN.)

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A better understanding of the relationships between insects and microfungi could help to identify the unknown factors reducing yields in maize. As the first step in current research, the aim was to isolate the microfungal species that can be found in the larval cavity of the European corn borer (*Ostrinia nubilalis* Hbn. *Lepidoptera. Pyraustidae*) (ECB), one of the most important insect pests of maize. In this way, the scale of potential phytopathogens spread by intermediate hosts could be reduced.

Fifty stalk sections damaged by ECB larvae were collected in autumn and fifty in spring on a 20-hectare plot in Ráksi (Somogy county). These were placed in wet chambers and incubated at room temperature under natural light. Identification was done from a pure culture inoculated into potato dextrose agar. Twenty-one species from 14 fungus genera were identified, the majority of which were mitosporic fungi. Species belonging to the *Fusarium, Acremoniella* and *Cladosporium* genera were predominant. Most of the species were saprotrophic, though some phytopathogenic species (*Gibberella, Colletotrichum, Nigrospora* and *Fusarium*) were also identified. The number of genera and the incidence of fungi were much higher in spring samples than in autumn ones, except for *Fusarium*, where incidence was lower in spring. It was found that failing to harvest the maize significantly enhanced the spread of several fungus species, especially phytopathogenic species, the following year, thereby serving as a source of infection.

Key words: maize, microfungal infection, European corn borer

Introduction

A great deal of research has been conducted on the biology of the pests and microfungi infecting maize, so the mechanism and extent of damage is well known. The yield loss caused by pests and microorganisms is referred to as primary or "direct" damage (Békési and Hinfner 1968; Kahler et al., 1985; Fitt, 1989; Mesterházy et al., 1979; Pálfy, 1983). In addition, if saprotrophic or phytopathogenic microorganisms become established "indirect" damage may appear, aggravating that caused by pests and causing further yield loss (Hatcher et al., 1997; Lodos, 1979; Hertelendy, 1999; Szeőke, 2003; Virág, 1959).

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In the case of maize this indirect damage is caused by microfungi (Ubrizsy, 1965; Fischl, 1995). Microfungal spores and the distribution of microfungal genera in the airspace of maize fields was examined by Fischl (1983). Several studies focused on the spore concentrations of different canopy levels and proved a connection between spore concentration and the extent of plant infection (Kramer et al., 1968; Van der Plank, 1967). As well as air-borne dispersal, microfungi were also found to be dispersed by pests (Gibbs and Inman, 1991). Ruming et al. (2004) proved the connection between *Sitophilus zeamalis* damage and *Aspergillus flavus* infection in maize, while Horváth and Vecseri (2004) identified the connection between *Helicoverpa armigera* and *Rhizophus* spp. infection in sunflower.

The number of pest vectors is increasing continuously, but little is known about the disease-carrying capacity of individual species or about the role of newly occurring pest species as vectors. In order to examine the role of vectors in disease epidemics, more information is required on the dispersal mechanisms of the particular pest (Hajek and St.Leger, 1994; Hatcher, 1995; Hatcher et al., 1997). The vector phenomenon has been examined mainly in the case of viruses, but this information could also help us to understand the development and dispersal of fungal diseases. It is not yet clear whether bacteria and fungi can occur and persist like viruses (Paine et al., 1997). A further question is whether, under certain environmental conditions, these phytopathogenic microorganisms might act as facultative entomopathogens.

Dispersal via insects was proved for two bacterial species (*Pantoea stewartii, Erwinia chrysanthemi*) (Elliot and Poos, 1940). It is possible that microfungal species may be spread by insects in a similar way, because the average dimensions of certain microfungal spores (mainly conidiospores) are similar to those of the *Erwinia* bacteria (2 μm) (Straub, 1978; Bánhegyi et al., 1985).

The insect–microfungi relationship raises several questions, the study of which could lead to a better understanding of yield loss in maize. As a first step, the aim of the present work was to isolate the fungi occurring in the larval cavities of *Ostrinia nubilalis*, in order to determine the phytopathogenic microfungal species potentially spread by this insect.

Materials and methods

In order to examine the relationship between *Ostrinia nubilalis* Hbn. and fungi, infected maize stalks were collected in a 20 ha maize field near the village of Ráksi (Somogy County, Hungary) on two occasions (25 October 2006, 11 March 2007). Samplings were planned for autumn and the following spring in order to obtain information on the seasonal appearance of the fungi. At each sampling date a total of 50 infected stalks exhibiting *Ostrinia nubilalis* damage were collected which, when cut, revealed the larval cavity.

The stalk pieces were cut longitudinally and placed in 19 cm diameter Petri dishes containing filter papers moistened with distilled water. Mosquito netting was placed on the filter papers to prevent the stalk pieces from coming into contact with the wet paper. Incubation was carried out at room temperature (21°C±1) in a wet chamber in natural light, without artificial illumination (Fig. 1). The mycological evaluation of the samples was started after 48 hours of incubation and continued for a week. Pure cultures of the fungi detected after incubation were grown on potato dextrose agar. The fungi were determined on the basis of spore characteristics.

The dead larvae or pellicles occurring in the larval cavities were first examined under the microscope, after which potato dextrose agar cultures were made to reveal the endogenous fungi. Determination was based on the works of Ubrizsy (1965), Ellis (1971; 1976), Booth (1971), Barnett and Hunter (1972) and Fassatiova (1984) using the nomenclature of the Index Fungorum (www.indexfungorum.org).

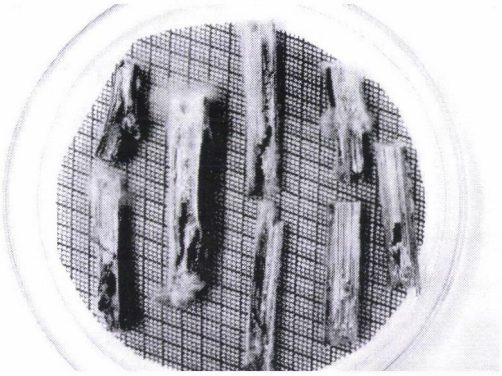

Fig. 1. Incubation of maize stalk segments in Petri dishes in a moist chamber

Results and discussion

A total of 21 fungal species belonging to 14 genera were detected in stalks damaged by *Ostrinia nubilalis* larvae (Table 1).

Table 1
Fungi species/genera isolated from maize stalks

Genera	Species
Mucor	*Mucor* sp.
Rhizopus	*Rhizopus oryzae* Went & Prins. Geerl.
	Rhizopus stolonifer var. *stolonifer* (Ehrenb.) Vuill.
Gibberella	*Gibberella zeae* (Schwein.) Petch
Colletotrichum	*Colletotrichum graminicola* (Ces.) G.W. Wilson
Acremonium	*Acremonium* sp.
Penicillium	*Penicillium* spp.
Gonatobotrys	*Gonatobotrys flava* Bonord.
Trichotecium	*Trichothecium roseum* (Pers.) Link
Nigrospora	*Nigrospora oryzae* Berk. and Broome
Acremoniella	*Acremoniella atra* (Corda) Sacc.,
Cladosporium	*Cladosporium cladosporioides* (Fresen.) G.A. de Vries
	Cladosporium herbarum (Pers.) Link
Alternaria	*Alternaria alternata* (Fr.) Keissl.
Epicoccum	*Epicoccum nigrum* Link
Fusarium	*Fusarium graminearum* Schwabe
	Fusarium oxysporum Schltdl.
	Fusarium semitectum Berk. & Ravenel
	Fusarium moniliforme J. Sheld.
	Fusarium subglutinans (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas

The majority of the species were mitosporic fungi (formerly *Deuteromycota*) and the dominant species were *Fusarium*, *Acremoniella* and *Cladosporium* (Table 2).

Table 2
Distribution of genera isolated from maize stalks

Sample	Genera													
	Mucor	*Rhizopus*	*Gibberella*	*Colletotrichum*	*Acremonium*	*Penicillium*	*Gonatobotrys*	*Trichotecium*	*Nigrospora*	*Acremoniella*	*Cladosporium*	*Alternaria*	*Epicoccum*	*Fusarium*
Autumn	*	*	*	–	–	–	*	–	*	**	**	**	*	***
Spring	*	*	**	*	*	**	**	*	–	***	***	**	*	**

– not found; * sporadic occurrence; ** medium occurrence; *** dominant occurrence

In the spring samples a higher number of genera was found than in the autumn samples (13 genera in spring, 10 in autumn), suggesting an increase in both stalk diseases (Fischl and Halász, 1990) and mycotoxin content (Szécsi et al., 2005). One species, *Nigrospora oryzae*, occurred only in the autumn samples, while others (*Colletotrichum, Acremonium, Penicillium, Trichothecium*) occurred only in the spring samples. A lower occurrence in spring was only observed for *Fusarium*. Three genera (*Mucor, Rhizopus, Alternaria*) exhibited no change in incidence, while four genera (*Gibberella, Gonatobotrys, Acremoniella* and *Cladosporium*) were more frequent in spring.

The majority of the species determined were saprotrophic. The phytopathogenic species belonged to the *Gibberella*, *Colletotrichum*, *Nigrospora* and *Fusarium* genera. The almost 100% infection with *Fusarium* was not surprising because these species are polyphagous; they may be both soil inhabitants and weakness pathogens, so they may appear in any injury to maize. On the other hand, a significant difference was observed between the autumn and spring samples as regards incidence. The *Fusarium* genus was present in all the autumn samples, the dominant species being *F. oxysporum* and *F. graminearum,* though *F. semitectum*, *F. moniliforme* and *F. subglutinans* were also present. Although *Fusarium* species also occurred frequently in the spring sample, the intensity of infection was lower in comparison with the autumn samples. Perithecia of the sexual form of *F. graminearum* (*Gibberella zeae*) were determined in a higher proportion in the spring samples, while they were only present in one autumn sample (Fig. 2).

Colletotrichum graminicola, which causes a rare disease, anthracnosis, in maize, only occurred in one stalk in the spring sample (Fig. 3), while *Nigrospora oryzae* was only determined from a single stalk in the autumn sample (Fig. 4). *Acremonium, Penicillium* and *Trichothecium* were also observed only in spring samples.

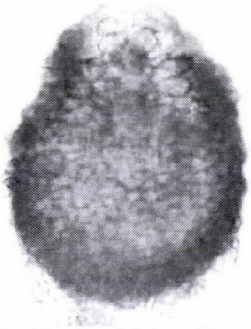

Fig. 2. Perithecia of *Giberella zeae*

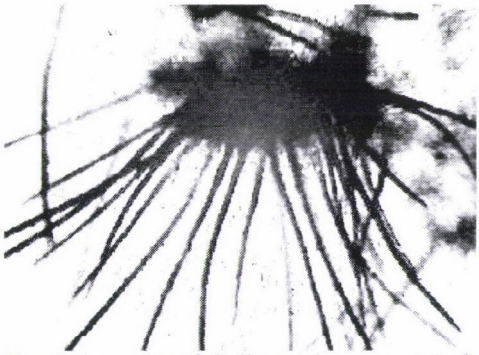

Fig. 3. Acervulus of *Colletotrichum graminicola*

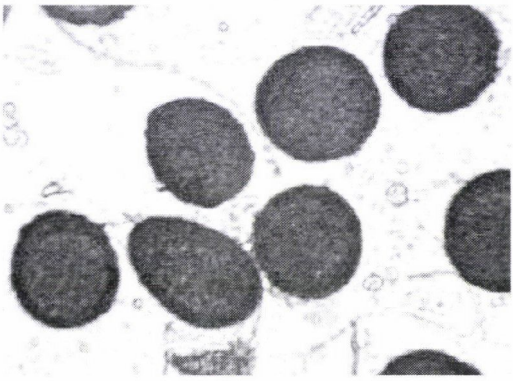

Fig. 4. Conidia of *Nigrospora oryzae*

The other saprotrophic species usually occur in decaying plant residues. It should be noted that *Acremoniella atra* and *Cladosporium* species were more frequent in the spring samples.

Only mycelia grew on the dead larvae and pellicles and no sporulation was observed, so the fungal species could not be determined.

In conclusion, unharvested maize plants may cause a significant increase in certain fungus species in the next vegetation period and could function as a source of infection.

As well as chewing the stalks, the larvae may also transmit phytopathogenic fungi, thus aggravating the damage and leading to higher yield losses.

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GENOTYPE AND YEAR EFFECTS ON MORPHOLOGICAL AND AGRONOMICAL TRAITS OF SILAGE MAIZE (*Zea mays* L.) HYBRIDS

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Leafy hybrids represent a new direction in the breeding of silage maize. Not only does the increased number of leaves above the ear in these hybrids lead to an increase in dry matter production, but the large quantity of carbohydrates formed and stored in the leaves results in silage with better chemical quality. Many papers have been published abroad on this subject, but few data have been reported in Hungary.

The present work aimed to examine the effect of genotype and year on six leafy and non-leafy silage maize hybrids over a period of four years (2002–2005), with special emphasis on the plant height, ear attachment height, leaf number, and fresh and dry matter yield.

The results showed that the number of leaves above the ear was much higher for the two leafy hybrids (8.00 and 9.35) than the average of the other hybrids (5.56, averaged over the years). This trait was in close negative correlation ($r^2 = -0.7346$) with the ratio of ear attachment height to total plant height, a trait with strong genetic determination, little influenced by the year. In leafy hybrids the main ear was located far lower down, but the total plant height was similar to that of the other hybrids. The ratio of ear attachment height to plant height was 0.36 for the leafy hybrids, but ranged from 0.41 to 0.45 for the other hybrids (averaged over the years). In wetter years the hybrids were taller and had greater dry matter production per plant than in the dry year.

Key words: silage maize, leafy, yield, genotype, year

Introduction

Silage maize production and the elaboration of silage-making techniques have a history of almost 150 years in Hungary. The main reason for the spread of this utilisation method was that, if the whole aboveground maize plant was utilised, a far larger quantity of feed could be harvested from unit area than if only the grain yield was used. The nutrient content of the whole maize plant is around one and a half times as much as that of the grain (Menyhért, 1985).

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The harvesting date has a substantial influence on the final digestibility and energy yield of silage maize. The plants are ripe for silage making when grain filling is complete but the plants are still green, with a dry matter content of 30–35% (Weissbach and Auerbach, 1999). However, silage maize can be harvested at a moisture content of 58% (42% dry matter) without any great loss, achieving 95% of the maximum yield per hectare (Darby and Lauer, 2002). According to Józsa (1981), if silage maize is harvested at a dry matter content of 35–40%, the ratio of the ear to the whole plant dry matter may be as high as 55–65%, which can be said to be a very good result. Maize harvested at this time also has better feed value than that harvested earlier, and this dry matter content is also satisfactory for the fermentation of the silage.

Carter et al. (1991) gave a very concise summary of the characteristics expected from a good silage hybrid: large dry matter yield, high protein content, high energy content, good digestibility, large water absorption ability (low fibre content), and optimum dry matter at harvest for satisfactory fermentation and storage.

The value of silage maize hybrids thus depends not only on the size of the fresh and dry matter yields per hectare, but also on the chemical quality and digestibility of the silage (Hunter and Bizard, 1988). Experiments have been underway all over the world in recent years to improve these parameters through the introduction of new genes such as *BMR* (brown midrib), *wx* (waxy), o_2 (opaque) and *floury-2*, and the use of hybrids with high oil content, all of which have led to considerable improvements in quality (Cox and Cherney, 2001). Their use in practice, however, has been restricted due to their poorer yield averages and to the negative effect of these genes on other agronomic traits. The use of leafy hybrids containing the *lfy1* gene, on the other hand, has proved to be favourable in the long term. This gene, discovered by R. C. Muirhead, was first described in detail by Shaver (1983), who reported on the origin and inheritance of the gene and on its generally positive effects on the morphology and yield of maize.

The main characteristic of leafy hybrids is that they have more leaves than normal hybrids, particularly above the main ear (Pintér et al., 1999). In addition, the ear attachment height is lower, the internodes are shorter, the stalk below the ear contains more lignin and the plants have greater yield potential. The increased leaf number above the ear results in a greater assimilating leaf area, allowing the plants to harvest the light energy required for photosynthesis more efficiently and thus to produce more nutrients in the leaves (Dwyer et al., 1995). Several authors have noted the positive effects of this on the yield and on grain quality (Stewart and Dwyer, 1993; Begna et al., 2001; Modarres et al., 1997; Dijak et al., 1999). The higher ratio of the leaves in the total plant dry matter and the greater carbohydrate content of the leaves above the ear (Andrews et al., 2000) have a favourable influence on the quality and digestibility of the silage (Lauer et al., 2001). Experiments carried out in Hungary (Pintér et al., 1988) proved that, above a harvest index of 35%, the energy concentration of the whole plant is determined not by the grain ratio but by the quality traits of the vegetative organs. In other words, a larger leaf area above the ear results in better quality silage.

Kámasil, the first leafy silage maize hybrid registered in Europe in 2002, was developed in Martonvásár, Hungary, and has since been followed by several others.

Many papers have been published on leafy hybrids abroad (chiefly in Canada, the USA and France), but few data have been reported in Hungary (Hegyi et al., 2009).

The present work aimed to examine the effect of genotype and year on various leafy and non-leafy silage maize hybrids, with special emphasis on the plant height, ear attachment height, leaf number, and fresh and dry matter yield.

Materials and methods

Six leafy and non-leafy silage maize hybrids bred in Martonvásár were grown under irrigated conditions in Martonvásár in four years, 2002–2005 (Table 1). Sowing was carried out at a rate equivalent to 80,000 plants/ha, and the experiment was laid out in a random block design with four replications. The same agronomic conditions were applied in all four years.

The weather differed greatly during the experimental years, which included both hot, dry and cool, wet summers. After an average year in 2002, 2003 was much hotter and drier, and the plants were affected by atmospheric drought (annual rainfall was only 367 mm and 25 very hot days were recorded in August). This was followed by two cool, wet years. In 2004 there were only two very hot days, in July, while in 2005 the rainfall sum was twice that recorded in 2003, and a large proportion of this (185.9 mm) fell in August. The total heat sum during the vegetation period was also higher by August in the first two years, amounting to 1405 and 1486, respectively, while in the third and fourth years these values were only 1206 and 1241 (based on data recorded in Martonvásár). These differences between the years were manifested in the phenotype of the plant stand and in the measured parameters.

Measurements were made on the plant height (from the ground to the tip of the tassel), the attachment height of the main ear, the ratio of ear attachment height to plant height, the leaf number below the ear, above the ear and in total, and the fresh and dry matter yield per plant, from which the dry matter yield per hectare was calculated. In addition, data were recorded on emergence (days from sowing), initial development, date of tasselling and silking, and proterandry.

One important aim was to reveal correlations between the various traits, especially in leafy hybrids, in order to determine to what extent other morphological and agronomic traits were influenced by the larger number of leaves above the ear.

The data were analysed with the Agrobase statistical program, using analysis of variance and correlation analysis based on the method of Sváb (1967).

Table 1
Hybrids tested in the experiment

No.	Hybrid	Vegetation period	Type
1	Limasil	FAO 380	Leafy
2	MvNK333	FAO 390	Non-leafy
3	Mv434	FAO 440	Non-leafy
4	Mv448	FAO 450	Non-leafy
5	Mv437	FAO 480	Non-leafy
6	Kámasil	FAO 510	Leafy

Results

Differences between the years were extremely noticeable for some traits (emergence, fresh and dry mass), while for others (ratio of ear attachment height to plant height) there were greater differences between the genotypes. In the case of tasselling, silking and dry matter content the genotype × year interaction was also strongly significant (Table 2).

Correlation coefficients (r) were significant at the p=0.1% level in all cases, with one exception, which is marked in the text.

Table 2
Significance of genotype, year and genotype×year effects for each trait

Trait	MS genotype	MS year	MS genotype×year
Emergence	1.167 *	124.917 ***	0.550 NS
Initial development	8.342 ***	13.125 ***	1.592 NS
Tasselling	209.769 ***	962.927 ***	6.535 ***
Silking	148.460 ***	660.483 ***	5.299 **
Proterandry	10.867 ***	42.792 ***	2.783 *
Plant height	4334.917 ***	13730.252 ***	158.855 NS
Ear attachment height	1565.978 ***	2294.173 ***	82.166 NS
Ear attachment height/plant height	0.022 ***	0.001 NS	0.001 NS
No. of leaves below the ear	3.605 ***	20.583 ***	0.623 ***
No. of leaves above the ear	47.405 ***	2.071 ***	0.524 *
Total leaf number	37.850 ***	14.050 ***	1.193 *
Fresh mass per plant	35812.930 *	75360.777 ***	18100.426 NS
Dry matter production per plant	5301.067 **	8954.249 ***	803.938 NS
Dry matter content	48.659 ***	219.164 ***	43.684 ***

***, **, *: Significant at the p=0.5%, 1% and 5% levels, respectively; NS: non-significant

Emergence and initial development

Emergence (expressed as days from sowing) took place at approximately the same time within each year, but there were considerable differences between the years. There were 8–9 days from sowing to emergence in 2003 and 14 days in 2004. In 2002 and 2004 there were no differences between the genotypes, which all emerged on the same day.

In the case of initial development, there were differences both between the years and between the genotypes, but the genotype × year interaction was not significant. The poorest values were recorded in 2002 and the best in 2005.

The values of initial development exhibited a moderate positive correlation with the ear attachment height (r = 0.4243) and the number of leaves below the ear (r = 0.4783).

Tasselling, silking, proterandry

In the case of tasselling and silking, the genotype and year effects and the genotype × year interaction were all strongly significant ($p<0.1\%$). MvNK333 flowered earliest, except in 2005 (Mv448), while the latest flowering genotypes were Mv437 and Kámasil in both years. The order of the other hybrids was variable. There was a very strong positive correlation between tasselling and silking ($r = 0.9670$).

In the case of proterandry, the order of hybrids was not as consistent. In 2004, for example, silking preceded tasselling in all cases, while in 2005 the opposite was observed, indicating that proterandry is extremely year-dependent. The genotype also had a significant influence.

Plant height, ear attachment height, ratio of ear attachment height to plant height

Both the plant height and the ear attachment height were significantly influenced by the year and the genotype, but only the genotype had a significant effect on the ratio of the two (Fig. 1).

The shortest (MvNK333) and tallest (Mv437) plants were the same in all the years, and the ear attachment height was also the lowest (Limasil) and the highest (Mv437) for the same hybrid. A close positive correlation was found between the two traits ($r = 0.7295$).

The ratio of ear attachment height to plant height was much smaller for leafy hybrids, being 0.36 for both, while the values ranged from 0.41–0.45 for the other hybrids, averaged over the years ($LSD_{5\%} = 0.0162$).

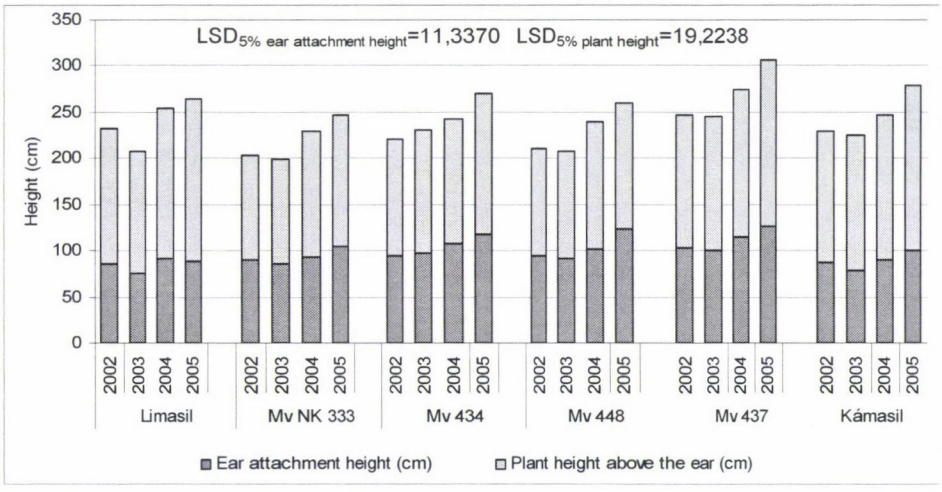

Fig. 1. Plant height and main ear attachment height of the hybrids in four years

No. of leaves below and above the ear, total leaf number

For all three traits, the effects of genotype and year were both strongly significant.

There was no significant difference between the leafy and non-leafy genotypes as regards the number of leaves below the ear, but the genotype × year interaction was significant (Fig. 2).

The greatest difference between the leafy and non-leafy genotypes was observed for the number of leaves above the ear. The extreme values were recorded for Mv448 (4.86) and Kámasil (9.35).

A close positive correlation (r = 0.8091) was observed between the number of leaves above the ear and the total leaf number, but these were not correlated with the number of leaves below the ear.

A close negative correlation was found between the number of leaves above the ear and the ratio of ear attachment height to plant height (r = –0.7346), while the correlation with the ear attachment height was only moderate (r = 0.4343).

The difference between leafy and non-leafy genotypes could also be observed for the total leaf number, but this difference was not as large. As there was a close correlation with the number of leaves above the ear, a correlation was also found with the ratio of ear attachment height to plant height (r = –0.5532). In addition, the total leaf number also exhibited a weak to moderate positive correlation with plant height (r = 0.4002).

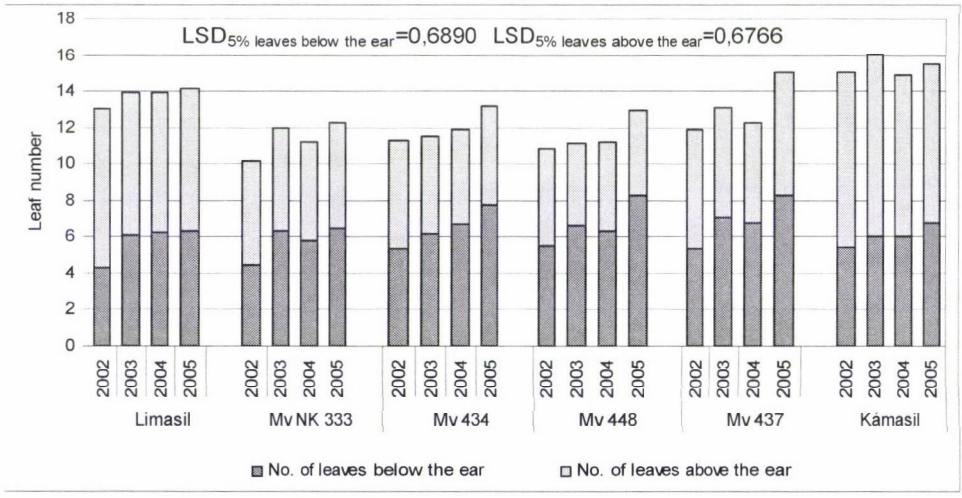

Fig. 2. Leaf number below and above the ear of the hybrids in four years

Fresh mass, dry mass, dry matter content

The dry matter production was strongly influenced by the genotype. The correlation between fresh mass per plant and dry matter was close and positive ($r = 0.7481$).

The dry matter content varied greatly, from 28 to 43%, between both genotypes and years and exhibited a moderate negative correlation with the fresh mass ($r = -0.4311$).

The fresh mass also exhibited a moderate correlation with the plant height ($r = 0.4165$), i.e. taller plants produced greater fresh mass.

The fresh mass per plant of the hybrids in the wet year (2005) was considerably greater than in the other years. The fresh mass of Kámasil, which had the greatest number of leaves above the ear, was slightly higher in 2002 and 2005 than that of the other hybrids, though this difference was not statistically significant (Table 3).

Both the fresh and the dry mass were weakly correlated with the number of leaves above the ear and the total leaf number. The dry mass (dry matter yield) and the total leaf number had a correlation coefficient of $r = 0.3121$ (significant at the p=0.5% level).

Table 3
Fresh mass per plant of the hybrids in four years (g per plant)

Hybrids	2002	2003	2004	2005	Year mean
Limasil	636.98	598.50	605.35	809.57	662.60
Mv NK 333	602.13	737.61	680.85	793.69	703.57
Mv 434	667.74	640.69	669.84	738.58	679.21
Mv 448	629.91	739.80	787.87	625.34	695.73
Mv 437	715.40	712.30	813.27	950.92	797.97
Kámasil	685.05	654.65	671.06	793.95	701.18
Hybrid mean	656.20	680.59	704.71	785.34	706.71

$LSD_{5\%\ genotype}$= 80.0378; $LSD_{5\%\ year}$= 65.3506; $LSD_{5\%\ genotype \times year}$= 160.0757

Discussion

It could be seen from the results that the year had the greatest influence on the flowering time, the proterandry and the fresh mass per plant and the least influence on the ratio of ear attachment height to plant height.

In general it can be said that in cooler, wetter years the plants were taller and their fresh mass was greater than in the dry year of 2003, i.e. their genetic potential was more clearly manifested. This confirms the findings of Józsa (1981), who reported that plant growth was retarded in the case of water deficiency, and yields were lower.

Plant samples were taken at 40 days after flowering in every year. The number of heat units accumulated to this date was different for the individual hybrids, which resulted in considerable differences in dry matter content.

The ratio of ear attachment height to plant height proved to have strong genetic determination and was less influenced by the year than the absolute values of plant height and ear attachment height. In the case of leafy hybrids, the ear was attached considerably lower compared to the total plant height, in agreement with earlier data (Shaver, 1983).

The leafy hybrids had substantially more leaves above the ear than the non-leafy hybrids, as also reported by other authors (Shaver, 1983; Dijak et al., 1999). It was reported in the literature (Dwyer et al., 1998) that the greater number of leaves above the ear resulted in greater fresh mass, but in the present experiment this difference was not significant for the given hybrids.

In recent years, new, up-to-date leafy hybrids have been developed in Martonvásár, and the latest results indicate that the above correlation is true for these hybrids. The increased nutrient content produced by the greater leaf area has also been found to have a favourable effect on the chemical quality (Hegyi et al., 2009).

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Short communication

SIMULTANEOUS WATER WITHHOLDING AND ELEVATED TEMPERATURE ALTERS EMBRYO AND ENDOSPERM DEVELOPMENT IN WHEAT

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Drought-tolerant Plainsman V and drought-sensitive Cappelle Desprez winter wheat genotypes were subjected to heat stress at 34/24°C combined with water withholding during early seed development in order to identify the joint effect of the stressors on embryo and endosperm development. During and after five days of treatment histological observations were made on the developing kernels and compared to yield data. Combined stress shortened the duration of the grain fill. With regard to kernel abortion, thousand-kernel weight and yield per spike, Plainsman V tolerated simultaneous elevated temperature and water withdrawal better than Cappelle Desprez. As a consequence of the stress the accumulation of B-type starch granules was almost completely absent in the endosperms of the sensitive genotype. The results indicate that compared to the drought-sensitive genotype, the tolerant genotype also showed increased tolerance of simultaneous drought and heat stress.

Key words: heat stress, embryo, endosperm, development, wheat

Introduction

The simultaneous occurrence of water deficiency and elevated temperature during the early generative stages of cereal ontogeny used to be rare, but due to the increasingly frequent occurrence of early season temperature extremes in many areas, more research may be justified. Despite the fact that drought and heat stress often coincide under field conditions and interact during the grain filling period of cereals, there is only limited information on the combined effect of these stress factors on kernel development. The combination of high temperature and drought reduced the duration of grain-fill more than either treatment alone in wheat (Nicolas et al., 1985; Altenbach et al., 2003; Shah and Paulsen, 2003). Interactions between the two stresses were pronounced, and the consequences of drought on all physiological and

developmental parameters were more severe at high temperature than at low temperature (Altenbach et al., 2003). The synergistic interactions indicated that the productivity of wheat is reduced considerably more by the combined stresses than by either stress alone, and that much of the effect is exerted on photosynthetic processes (Shah and Paulsen, 2003). However, the combined effects of heat and drought are not necessarily additive ones. For example, in the case of kernel dry weight at maturity, high temperature reduced the effect of post-anthesis drought in wheat. It was suggested that where high temperature and drought occur concurrently after anthesis, a degree of drought escape may be associated with chronic high temperature because of the reduction in the duration of grain filling, even though the rate of water use may be enhanced by high temperature (Wardlaw, 2002). On the other hand, under certain circumstances, limited water availability may promote nutrient remobilisation from the leaves and stems and increase the rate of grain filling (Yang and Zhang, 2006), thus compensating for its shortened duration.

Materials and methods

The plants were grown in phytotron chambers in a controlled environment. The spikes were isolated prior to anthesis, and pollination was carried out with control pollen. The control temperature was 23/14°C. Stress treatment involved heat stress at 34/24°C combined with water withholding for 5 days during early seed development (1–5 days after pollination, DAP). After the stress treatment the plants were returned to the control chambers with a temperature of 23/14°C and daily water supplies of 150 ml, as for control plants. The plants were grown to full maturity and yield components were evaluated. Statistical analysis was carried out using a balanced analysis of variance (ANOVA). For light microscopy 1, 2, 3, 4 and 5-day-old kernels were fixed for 4 h at room temperature in primary fixative containing 2.5% (v/v) glutaraldehyde in 0.05 M Na-cacodylate buffer, pH 7.2. After fixation, the samples were washed, dehydrated, infiltrated with Unicryl embedding medium, and polymerised for 10 days by UV irradiation. Semithin sections were cut using an Ultracut E microtome and mounted on microscopic slides. The sections were stained with periodic acid-Schiff (PAS) for polysaccharides and Coomassie Brilliant Blue for proteins, and examined using an Olympus BX51 microscope.

Results and discussion

Reduction in yield

Similarly to the results of Nicolas et al. (1985), Altenbach et al. (2003) and Shah and Paulsen (2003), the grain-filling period of stressed kernels was one week shorter in both genotypes compared to the control. The kernel abortion increased significantly in stressed plants (Table 1) and the increment was greater in the drought-sensitive genotype. The thousand-kernel weight and the yield per spike were severely reduced, especially in the upper third of the spikes (data not shown). Thousand-kernel weight decreased markedly in plants subjected to combined stress, by 71.5% and 35.7% in sensitive and tolerant genotypes, respectively. As regards the yield per spike, drought-tolerant Plainsman V tolerated simultaneous elevated temperature and water withdrawal better than drought-sensitive Cappelle Desprez, with values 75% higher (Table 1). It can be stated for all the parameters investigated that Plainsman V responded to combined heat and drought stress more tolerantly than Cappelle Desprez.

Table 1
Yield characteristics of drought-tolerant Plainsman V and drought-sensitive Cappelle Desprez plants exposed to simultaneous drought and heat stress during early seed development

Genotype	Kernel abortion	1000-kernel weight	Yield/spike
	as a % of the control		
Cappelle Desprez	38.4	28.5	16.3
Plainsman V	25.2	64.3	65.0

Histological alterations

Combined heat and drought stress accelerated embryo development significantly until 5 days after fertilisation (DAP), when the embryos in treated Cappelle Desprez and Plainsman V kernels were 8 and 11 times larger than in the control, respectively (Fig. 1, Fig. 2B, D). Compared to the sensitive genotype, the tolerant genotype responded earlier to the combined stress, so embryos developing in Plainsman V kernels were significantly larger ($P < 0.005$) than Cappelle Desprez embryos (Fig. 1).

Combined stress also affected endosperm development and the maternal cell layers surrounding the filial tissues during treatment in both genotypes. The consequences of accelerated development are demonstrated in Fig. 2. The senescence of the outer integument, usually observed in control kernels at 3 DAP, was perceptible as early as 1 DAP in treated kernels. By the 5th day of development the interior layer of the inner integument started to accumulate resinous compounds (Fig. 2E–H) and the nucellar epidermis present in control kernels started to disintegrate (Fig. 2E–H). These features are usually typical of control kernels at 7–9 DAP. The accumulation of A-type starch granules, which is typical of the 5th day (Fig. 2I–L), was observed in the endosperms of 3-day-old kernels. The accumulation of starch reserves was accelerated in the treated endosperm cells (Fig. 2J–L).

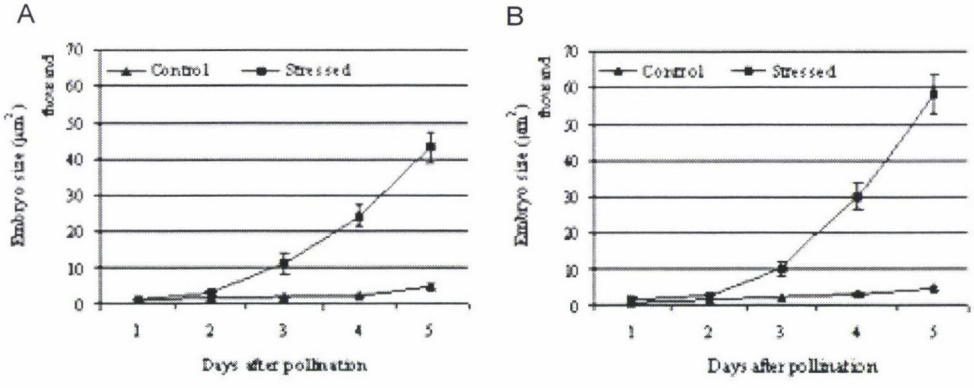

Fig. 1. Embryo size in control and stressed Cappelle Desprez (A) and Plainsman V (B) kernels between the 1st and 5th days of development

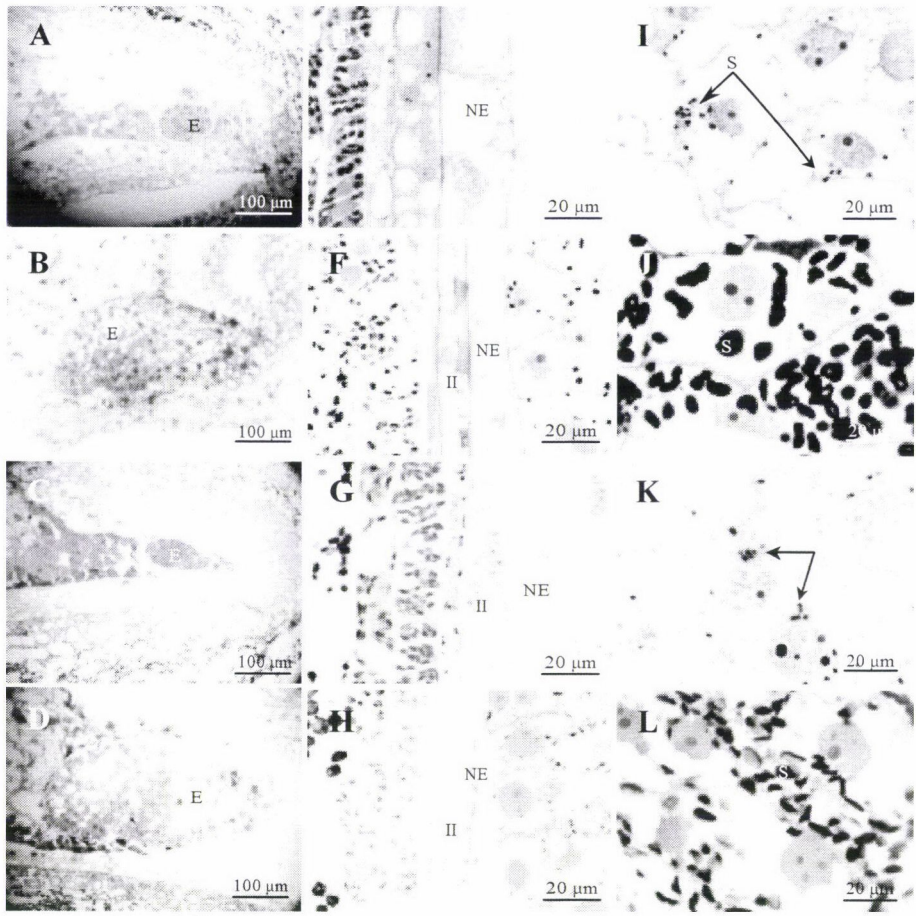

Fig. 2. Light micrographs of Cappelle Desprez (control: A, E, I; stressed: B, F, J) and Plainsman V (control: C, G, K; stressed: D, H, L) embryos (A–D), cell layers surrounding the filial tissues (E–H) and endosperms (I–L) on the 5th day of embryo development. E: embryo, II: inner integument, NE: nucellar epidermis, S: starch granules

Although the plants were re-watered at 5 DAP, the partially regenerated vegetative tissues were unable to provide the required nutrient supplies to the rapidly developing filial tissues. The depression in yield was a consequence of the reduced nutrient supplies and the shortened duration of grain fill. Scanning electron micrographs showed that, while in the stressed endosperms of the tolerant genotype the size and distribution of A- and B-type starch granules was similar to the control (Fig. 3A, B), in treated endosperms of the sensitive genotype the accumulation of B-type starch granules was almost completely absent (Fig. 3C, D). In conclusion, the results indicate that compared to the drought-sensitive genotype, the tolerant genotype also showed increased tolerance of simultaneous drought and heat stress.

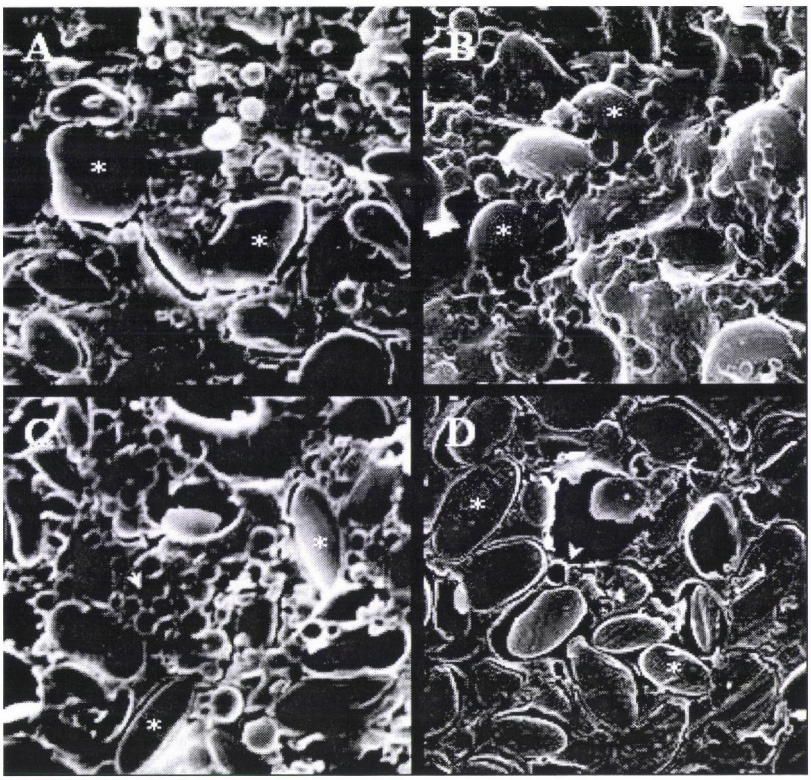

Fig. 3 Scanning electron micrographs of A- (asterisk) and B-type (arrowheads) starch granule distribution in control (A, C) and treated (B, D) endosperms of drought-tolerant Plainsman V (A, B) and sensitive Cappelle Desprez (C, D) winter wheat genotypes

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Short communication

EXAMINATION OF CHEMICAL COMPOSITION AND CALORIFIC VALUE OF CEREAL STRAW

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Representative straw samples from various cereals were analysed to determine their chemical composition and calorific value. It was found that the chemical composition data given in previous feeding tables can be applied to characterise modern varieties, as only the crude fat contents of oats and winter wheat were significantly higher than the available reference data, while the ash contents were lower. The calorific value of cereal straw was equal to or in some cases greater than that of energy grass, so cereal straw could be competitive with energy grass, due to its large cultivation area and the properties and value of its by-products.

Key words: cereal straw, chemical composition, calorific value

Introduction

Straw is a by-product of cereal production and is produced every year in large and relatively predictable amounts (Užik and Žofajová, 2006). Nowadays it is primarily used as animal bedding, but its chemical composition makes it suitable for use in other industries, to increase the fibre content of feed mixes or, due to its high cellulose content, in the manufacture of cellulose or chipboard (Puls, 1993). If hay is scarce it can even be used as forage, and research is increasingly aimed at utilizing the energy stored in chemical bonds in the bio-energy industry (Epstein et al., 1978).

The chemical composition of the straw from various cereal species exhibits only slight differences. The average protein content is 3.1–4.1%, the crude fat content 1.6–2.0%, the crude fibre content 43.2–47.2% and the ash content 4.7–6.5%. Schmidt (1993) reported the highest protein content for winter wheat straw, the highest crude fat content for oat straw, the highest crude fibre content for rye straw and the highest ash content for winter barley, and the limit

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values were found to be the same as those given by Kakuk and Schmidt (1988). However, other authors reported wider limits, the values of the ash content of winter wheat being given as 7.5–8.5 % (Ali et al., 1991), 4.0–9.0% (Utne and Hegbom, 1992) and 3.7% (Misra, 1987). The ash content and composition were presented in detail by Kádár et al. (2007). According to Utne and Hegbom (1992), this wide diversity is due to the fact that the ash content and chemical composition of straw are highly influenced by the soil and other environmental factors.

Research on the heat energy released from chemical bonds by combustion is of increasing importance, because of the large quantity of straw arising as the by-products of crop production (including maize) on about 3 million hectares, combined with the continuous decrease in the volume of animal production, as the primary utilization sector (Boateng et al., 2007). Although it cannot fully substitute energy grass in energy production, the fact that it has a similar specific energy yield (Janowszky, 2003) and is relatively inexpensive could make cereal straw a useful source of energy.

The aim of this study was to determine the calorific value and chemical composition of various types of cereal straw grown in Hungary and to check the accuracy of reference data.

Materials and methods

Straw from six cereal species (winter wheat, winter barley, rye, oats, triticale and spelt) and from two other plants (grass and rape) was collected for the purposes of comparison in 2007. Samples were taken after natural drying from randomly selected fields in Hajdú-Bihar, Borsod-Abaúj-Zemplén and Szabolcs-Szatmár-Bereg counties, and from experimental plots at the Experimental Station of the University of Debrecen in Látókép. Ten samples were taken for winter wheat, eleven for winter barley, eight for rye, four for triticale, eight for oats, two for spelt, two for grass and four for rape.

The quality was analysed in the accredited laboratory at the University of Debrecen. The plant samples were dried and ground in a Retsch SR-200 Rotor Mill (Haan, Germany) to pass through a 1 mm sieve. The protein content was determined by the Kjeldahl method, the crude starch content by enzymatic determination, the crude fibre content by the intermediate filtration method, the ash content by incineration 550°C and the crude fat content by diethyl ether extraction. The calorific value was recorded using an IKA WERKE C2000 (Staufen, Germany) calorimeter. The statistical analysis was done using the SPSS 12.0 program package.

Results and discussion

The chemical composition of the straw samples is presented in Table 1. The protein content in the straw of all the different cereal species was similar (3.1–4.2%) and was close to the Hungarian reference values. The standard deviation was relatively low (0.14–0.65%), demonstrating that the variability in the protein content between different cropping sites was much lower for the straw than for the grains. Similar results were obtained for the raw fibre content: the standard deviations and coefficients of variation were relatively low (0.63–

1.6% and 1.6–4.0%, respectively), and the averages were similar for the different cereal species (40–45%). The non-cereal species had higher protein content than the cereals, but the raw fibre content of the grass was only 58–66% that of cereal straw.

The crude fat content exhibited greater deviation, with the highest value for oats (2.59% on average), followed by winter wheat and spelt (2.29 and 2.09%, respectively). The crude fat content of the other cereals was similar, ranging between 1.55 and 1.65%. The relatively low standard deviation indicated weak genotype and environmental effects. The fat content in the straw of grass and rape was similar to that of cereals. The ash content of the cereal straws was similar for all the species (4.5–5.7%). Grass had significantly higher values, while the ash content of rape straw was lower.

The highest calorific values were found for triticale and rye (18 800 and 18 763 J g^{-1}) and the lowest for oats (just below 18 000 J g^{-1}). The standard deviation was relatively low, indicating moderate genotype and environmental effects, except for winter barley, where the calorific values ranged from 17 500 to 19 770 J g^{-1}. The average calorific value of rape straw was similar to that of cereals, but much lower values were measured for grass (16 080 J g^{-1}). The results show that cereal straw could be a promising raw material for energy production, as the calorific value of energy grass, produced specifically for this purpose, is considerably lower (14 000–17 000 J g^{-1}) (Janowszky, 2003). Considering that the calorific value of coal ranges from 15 000–27 000 J g^{-1}, that of lignite is about 8 000 J g^{-1} and that of lignite briquette about 20 000 J g^{-1}, cereal straw could be a substitute for these fuels in the vicinity of the cropping sites.

Table 1

Crude protein, fibre, fat and ash contents and calorific value of straw samples, 2008 (% in dry matter)

Straw samples	Crude protein content	Crude fat content	Crude fibre content	Crude ash content	Calorific value
Winter barley	3.52 abc	2.29 b	45.15 b	5.32 bc	17979 a
Winter wheat	4.16 c	1.56 a	40.71 a	5.33 bc	18383 a
Rye	3.68 bc	1.68 a	39.92 a	5.07 bc	18764 a
Triticale	3.18 ab	1.68 a	43.29 b	4.51 ab	18799 a
Oats	3.67 bc	2.60 bc	40.39 a	5.26 bc	17842 a
Spelt	2.86 a	2.90 c	38.28 a	5.57 c	18191 a
Grass	5.70	1.65 a	26.36	7.20 d	16080
Rape	4.92	1.74 a	52.42	3.76 a	18660 a

Means followed by the same letter within columns were not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

When estimating the economic potential, the annual quantity of straw available per unit must be taken into account as well as the calorific value. In 2007 winter wheat was grown on the largest area in Hungary (926 000 ha), but the areas of other cereals are also significant (barley 271,000 ha, triticale 92,000 ha, oats 39,000 ha, rye 26,000 ha). Nowadays only about 56–59% of the straw produced in Hungary is harvested, while the rest is burnt or ploughed in (Fig. 1). Almost 50% of the straw produced (approx. 2 million tonnes for winter wheat in 2007) could thus be used for energetic purposes. According to the available data, this amount would provide roughly 27.75 PJ energy, sufficient to cover 2.4% of Hungarian primary energy consumption. Considering the average price of natural gas (8.26 thousand euro/GJ in 2007) 237 thousand million euro per year could be saved by burning straw.

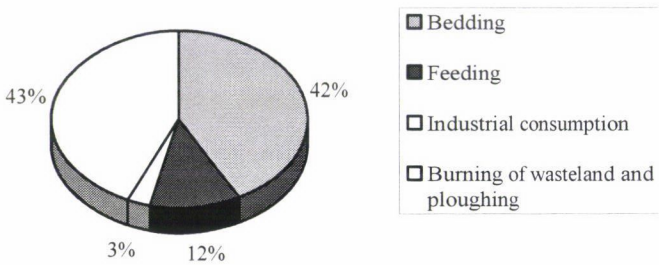

Fig. 1. Straw utilisation in Hungary (Barótfi, 1998)

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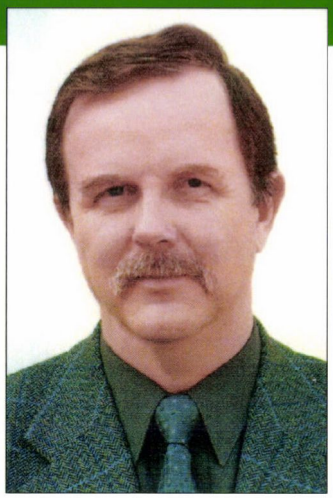

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PHYSIOLOGICAL EFFECTS OF GLYCINEBETAINE ON GAMMA-IRRADIATED STRESSED FENUGREEK PLANTS

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Irradiation stress adversely affects plant growth and development. No radio-protective activity of glycinebetaine (GB) has yet been reported in plants. When applied pre-sowing to dry seeds of fenugreek, gamma rays at doses of 0, 25, 50, 100 and 150 Gray (Gy) from a cobalt (^{60}Co) source with a strength of 500 Ci and a dose rate of 0.54 Gy/min significantly reduced the chlorophyll content, total protein, photosynthetic efficiency ($^{14}CO_2$ fixation), total dry weight, and accumulation of reducing, non-reducing and total soluble sugars in comparison with the un-irradiated control. It also significantly repressed the activities of hydrolytic enzymes (α-amylase and invertase) and the carboxylating enzyme (ribulose-1,5-bisphosphate-carboxylase/oxygenase) in the fenugreek plants. Soaking irradiated seeds with glycinebetaine (50 mM) for 24 hours partially alleviated the depression effects of irradiation in these parameters. Gamma irradiation significantly increased the H_2O_2 content, while pre-soaking irradiated seeds with GB significantly decreased the H_2O_2 level. The magnitude of the reversal declined as the irradiation dose increased.

Gamma irradiation induced a significant decrease in the level of nucleic acids (DNA and RNA), accompanied by a corresponding induction of the hydrolytic activities of DNase and RNase in comparison with the un-irradiated control. These changes were more significant at higher γ-ray doses. Post-treatment of irradiated seeds with GB partially alleviated the adverse effects of radiation, significantly increasing nucleic acid levels and repressing the activities of DNase and RNase. The protective role played by glycinebetaine was more significant at lower γ-ray doses. Pre-treatment of seeds with GB may play an effective role in the radio-repair mechanism.

Key words: glycinebetaine, photosynthetic efficiency, gamma irradiation

Abbreviations: GB: glycinebetaine; Rubisco: ribulose 1,5-bisphosphate carboxilase/oxygenase; Gy: Gray; RNase: ribonuclease; DNase: deoxyribonuclease

Introduction

Fenugreek (*Trigonella foenum-graecum*) is an important medicinal plant belonging to the Fabaceae family. It is used both as a herb (the leaves) and as a spice (the seed). It is cultivated worldwide as a semi-arid crop. Gamma

irradiation induces various physiological and biochemical alterations in plants (Seung et al., 2007). Changes in the carbohydrate metabolism in response to γ-irradiation were shown to be a function of photosynthesis (El-Fiki et al., 2003) and phytohormones (El-Aishy et al., 1976). The extent of plant responsiveness to irradiation varied with species/cultivar and the dose of irradiation (Kim et al., 2002). Enhanced doses of gamma irradiation reduced the carbohydrate content in potato (Joshi et al., 1990). Subjecting pea to different doses of gamma rays reduced the accumulation of reducing, non-reducing and total soluble sugars (Selim and El-Banna, 2001). Gamma irradiation reduced photosynthetic activity (Kim et al., 2005), and repressed the α-amylase activity in chick pea (Khanna and Maharchandani, 1985) and the invertase activity in wheat (Zhang and Mao, 1993).

It has generally been accepted that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen, are produced by water radiolysis (Kovács and Keresztes, 2002). Among these ROS, H_2O_2 is a normal metabolite in cells and is not particularly cytotoxic under optimal plant growth conditions, but when its concentration is increased by environmental stress or ionizing radiation, it can lead to cell lethality.

Ionizing radiation has long been recognized as a potent mutagen, and its interference with the nucleic acid metabolism is well documented (Kovalchuk et al., 2001). A linear inverse relationship was found to exist between irradiation dose and DNA/RNA content when barley seeds were irradiated with γ-rays (Joshi et al., 1970). Disturbance of the nucleic acid biosynthetic process was reported after seeds were irradiated with high doses of γ-rays (Seung et al., 2007). The available literature reveals the catabolism of DNA and RNA by different doses of γ-rays (Danylchenko and Sorochinsky, 2005). The activities of deoxyribonucleases, ribonucleases and nucleases in *Verticillium agaricinum* were enhanced by irradiation (Osman and Mohamed, 1985).

Glycinebetaine is a quaternary ammonium compound. It is non-toxic, highly water-soluble and readily absorbed in various tissues (Diaz-Zorita et al., 2001). GB is naturally biosynthesized under stressful conditions (Jagendorf and Takabe, 2001). Efforts are underway to metabolically engineer plants with enhanced capacity to accumulate GB (Quan et al., 2004). As an effective compatible solute, glycinebetaine is involved in the defensive responses of higher plants to extreme conditions of salt, drought, temperature or light stress (Wahid and Shabbir, 2005; Farooq et al., 2008). Rubisco is a bifunctional enzyme catalysing two competing reactions, photosynthetic CO_2 fixation and photorespiratory carbon oxidation, in chloroplast stroma. The enzyme accounts for 12–35% of total leaf protein (Makino et al., 1983). The irradiation of seeds with high doses of gamma rays disturbs the synthesis of protein (Xiuzher, 1994). GB stabilized Rubisco when added to a crude extract of rice seedlings that was subsequently heated to 50°C *in vitro* (Dionisio-Sese et al., 1999). However, some plant species lack the ability to synthesize GB in sufficient amounts (Yang et al., 2003), and its exogenous application becomes imperative to induce stress tolerance. Some studies acclaim the beneficial role of GB as foliar application and others as seed treatment (Naidu and Williams, 2004).

The phytoprotective effect of glycinebetaine against gamma irradiation stress in fenugreek has not yet been studied. The present research investigated the possible mediatory role of glycinebetaine in alleviating the harmful effect of gamma-irradiation stress. Physiological and biochemical changes were assessed in parameters associated with oxidative stress induced by γ-irradiation, namely total dry weight, H_2O_2 content, protein content, chlorophyll content, photosynthetic efficiency ($^{14}CO_2$ fixation), carbohydrate content, nucleic acid content and the enzyme activities of RNase, DNase, invertase, α-amylase and ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco).

Materials and methods

Plant material, growth conditions and stress treatments

A homogeneous lot of fenugreek seeds (*Trigonella foenum-graecum*) cv. Giza 2 was obtained from the Crop Institute, Agricultural Research Center, Giza, Egypt and stored at 4°C. The seeds were surface sterilized in 0.1% (w/v) sodium dodecyl sulphate solution and then thoroughly rinsed with sterile deionized water. The dry seeds were exposed to various doses of gamma irradiation, 0, 25, 50, 100 and 150 Gray (Gy), using a gamma source (^{60}Co) (Vinderen, Oslo, Norway) with a strength of 500 Ci and a dose rate of 0.54 Gy/min at the Middle Eastern Regional Radioisotope Center for the Arab Countries (Dokki, Cairo, Egypt). Irradiated and un-irradiated seeds were soaked for 24 hours in 50 mM glycinebetaine (Wako Pure Chemicals Co. Ltd., Osaka, Japan) solution or in distilled water. The seeds were placed on Whatman No. 1 filter papers moistened with full-strength Hoagland's nutrient solution in 15 cm Petri dishes and allowed to germinate in the dark at 24°C for three days (Rafi and Epstein, 1999). Selected healthy seedlings of equal size and vigour were transplanted to black polyethylene pots (10 cm high × 5 cm diameter containing continously aereated full-strangth Hoagland's nutrient solution. The plants were grown for one month in a controlled growth chamber under the following growth conditions: 15-h photoperiod; 65–75% relative humidity; day and night temperature of 22°C and 20°C. The photosynthetic photon flux density at maximum plant height was about 440 μM $m^{-2}s^{-1}$. After one month, the fenugreek plants were harvested for analysis.

Nucleic acid determination

DNA and RNA were extracted with 10% cold perchloric acid following the method of Kalinich et al. (1985).

Enzyme assay

Ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco, EC 4.1.1.39) was determined as reported by Warren et al. (2000). Invertase activity (EC 3.2.1.26) was assayed according to the method of Lavon et al. (1995) and α-amylase activity (EC 3.2.2.1) following the method of Davis (1977). Ribonuclease activity (RNase, EC 3.1.14.1) and deoxyribonuclease activity (DNase, EC 3.1.21.1) were determined according to the method of Lee et al. (1976) and Rasskazov et al. (1986), respectively.

Photosynthetic pigments

Total chlorophyll was determined according to the method of Lichtenthaler and Wellburn (1983).

Chemical analysis

Total soluble protein contents were measured using the method of Bradford (1976) and hydrogen peroxide according to Patterson et al. (1984). Reducing sugars were determined using salicylic acid as recommended by Miller (1959), total soluble sugars using 80% phenol as described by Dubois et al. (1956) and non-reducing sugars as the difference between total soluble carbohydrates and reducing sugars.

Photosynthetic activity ($^{14}CO_2$ fixation)

Photosynthetic activity ($^{14}CO_2$ assimilation) was measured in the Atomic Energy Authority Radioisotope Department, Cairo, Egypt, with the method of Moussa and Abdel-Aziz (2008). For each treatment one Petri dish containing the seedlings was placed under a Bell jar, which was used as a photosynthetic chamber. Radioactive $^{14}CO_2$ was generated inside the chamber by a reaction between 10% HCl and 50 μCi (1.87×10^6 Bq) $NaH^{14}CO_3$ + 100 mg Na_2CO_3 as a carrier. Then the samples were illuminated with a tungsten lamp. After 30 min exposure time, the leaves were quickly detached from the stem, weighed and frozen for 5 min to stop the biochemical reactions, then subjected to extraction by 80% hot ethanol. The ^{14}C was assayed from the ethanolic extracts in soluble compounds using a Bray Cocktail (Bray, 1960) and a Liquid Scintillation Counter (LSC2-Scaler Ratemeter SR7, Nuclear Enterprises, Edinburgh, UK).

Total dry weight

For dry weight determination, complete plants were dried in a hot air oven at 70°C to constant weight.

Statistical analysis

The data were statistically analysed using the least significant difference test (L.S.D.) at the 5 and 1% levels of probability (Steel and Torrie, 1980). The data were also subjected to analysis of variance for a split plot design using the statistical program Minitab.

Results and discussion

The substantial alterations induced by γ-irradiation in the soluble carbohydrate metabolism in fenugreek were restored by pre-sowing soaking treatment with GB. Gamma irradiation at 0, 25, 50, 100 and 150 Gy significantly reduced the concentration of soluble sugars (reducing, non-reducing and total soluble carbohydrates) along with the activities of α-amylase and invertase in the developed seedlings (Tables 1 and 2). The reduction in these parameters was more considerable at higher doses of gamma irradiation. The results are consistent with the findings of Lima et al. (2001), who ascribed the decrease in reducing and non-reducing sugars to the lower activities of invertase and amylase. Levitt (1980) attributed the decrease in soluble sugar levels to the inhibition of photosynthesis. Gamma irradiation was found to inhibit CO_2 assimilation (Stoeva, 2002) and to decrease the activity of α-amylase and invertase, the hydrolytic enzymes involved in the carbohydrate metabolism (Machaiah et al., 1976). The decrease in soluble sugar levels, along with that of α-amylase and invertase activities, may imply the depletion of GA_3 by γ-irradiation, which represses the activity of certain hydrolases (Gaber et al., 2000). The data presented in Table 1 revealed the positive significant influence of exogenous GB on the accumulation of soluble sugars and on the hydrolytic activities of α-amylase and invertase (Table 2) at different doses of γ-irradiation. GB was reported to enhance the accumulation of soluble sugars by accelerating the rate of photosynthesis (Yang and Lu, 2005). GB increased the seedling dry weight, soluble sugar content and α-amylase activity of maize (Farooq et al., 2008). GB was also shown to enhance the activity of invertase (Kawahara et al., 1990) and to

Table 1

Effect of various doses of γ-irradiation in the presence and absence of GB on chlorophyll *a*, chlorophyll *b*, total chlorophylls (*a*+*b*), photosynthetic activity, reducing, non-reducing and total soluble carbohydrates, H_2O_2 content and total dry weight in fenugreek plants after one month

Parameters	GB (mM)	γ-irradiation doses (Gy)				
		0	25	50	100	150
Chl. $a^{\#}$	0.0	1.28±0.02	1.22±0.03^{+}	1.03±0.04^{++}	0.83±0.02^{++}	0.54±0.02^{++}
	50	2.60±0.05**	2.14±0.06**	1.98±0.07**	1.98±0.08**	1.05±0.03**
Chl. $b^{\#}$	0.0	0.54±0.02	0.48±0.01^{+}	0.31±0.02^{++}	0.26±0.01^{++}	0.18±0.02^{++}
	50	1.32±0.03**	1.08±0.05**	0.93±0.03**	0.93±0.01**	0.39±0.01**
Total chl. $(a+b)^{\#}$	0.0	1.82±0.01	1.70±0.08^{+}	1.34±0.07^{++}	1.09±0.04^{++}	0.72±0.01^{++}
	50	3.92±0.11**	3.22±0.29**	2.91±0.23**	2.91±0.12**	1.44±0.88**
Photosynthetic activity$^{\#\#}$	0.0	10.23±0.82	9.62±0.96^{++}	7.13±0.42^{++}	5.02±0.25^{++}	3.36±0.18^{++}
	50	16.72±1.17**	12.06±1.21**	11.15±0.48**	9.18±0.18**	7.75±0.39**
Reducing sugars$^{\#}$	0.0	35.61±2.14	32.18±0.64^{++}	29.63±0.88^{++}	24.31±1.45^{++}	17.82±1.06^{++}
	50	46.13±3.15**	40.02±2.0**	36.18±1.81**	28.65±1.72**	25.07±1.25**
Non-reducing sugars$^{\#}$	0.0	16.82±1.34	16.93±0.34	13.07±0.65^{++}	9.15±0.64^{++}	4.28±0.26^{++}
	50	25.11±2.27**	19.48±1.55**	16.99±1.65**	13.82±1.52**	10.05±0.80**
Total soluble sugar$^{\#}$	0.0	52.43±3.67	49.11±3.92^{+}	42.70±3.81^{++}	33.46±2.67^{++}	22.10±1.10^{++}
	50	71.24±4.25**	59.50±5.8**	53.17±4.78**	42.47±2.55**	29.12±2.91**
Total dry weight$^{\#\#\#}$	0.0	0.125±0.003	0.129±0.002	0.092±0.001^{++}	0.063±0.003^{++}	0.040±0.004^{++}
	50	0.261±0.005**	0.215±0.001**	0.193±0.003**	0.152±0.005**	0.108±0.003**
$H_2O_2{}^{\#\#\#\#}$	0.0	6.23±0.15	7.14±0.18^{+}	11.28±0.23^{++}	14.35±1.15^{++}	20.26±1.82^{++}
	50	4.03±0.03**	5.13±0.26**	7.33±0.59**	9.05±0.91**	12.65±1.53**

$^{\#}$: (mg g FW^{-1}); $^{\#\#}$: (kBq mg FW^{-1}); $^{\#\#\#}$: (g $plant^{-1}$); $^{\#\#\#\#}$: (μM g FW^{-1}); $^{++}$ and $^{+}$ denote significant differences between γ-irradiated plants and controls at the 0.01 and 0.05% levels, respectively; ** and * denote significant differences between plants treated with γ-irradiation and plants treated with γ-irradiation+GB at the 0.01 and 0.05% levels, respectively. Data are the means of four replicates ± SE.

Table 2

Effect of various doses of γ-irradiation in the presence and absence of GB on the enzyme activities of Rubisco (mg g FW^{-1}), α-amylase (starch hydrolysed/min mg FW^{-1}), invertase (mg sucrose hydrolysed/min mg FW^{-1}), DNase and RNase ((mg g FW^{-1}) in fenugreek plants after one month

Parameters	GB (mM)	γ-irradiation doses (Gy)				
		0	25	50	100	150
Rubisco	0.0	3.3±0.03	3.0±0.09^{++}	2.1±0.10^{++}	1.5±0.06^{++}	1.1±0.08^{++}
	50	5.4±0.38**	3.8±0.16**	3.2±0.25**	1.9±0.09**	1.6±0.11**
α-amylase	0.0	56.4±4.5	57.0±5.1^{+}	40.8±2.5^{++}	32.5±2.3^{++}	21.0±1.0^{++}
	50	71.6±2.5**	63.8±5.1**	56.6±5.6**	50.2±6.0**	38.7±3.7**
Invertase	0.0	429±21	420±16^{++}	370±29^{++}	342±13^{++}	313±19^{++}
	50	536±16**	465±32**	452±27**	403±16**	362±21**
DNase	0.0	10.6+0.2	13.8±0.3^{++}	17.4±0.3^{++}	20.5±0.6^{++}	25.8±0.8^{++}
	50	8.4±0.7**	12.0±0.6**	14.5±0.7**	16.2±0.8**	19.9±1.2**
RNase (mg g FW^{-1})	0.0	25.6+2.1	30.1±0.5^{++}	43.2±1.3^{++}	60.5±6.6^{++}	68.8±4.8^{++}
	50	19.7±2.5**	23.8±2.3**	34.9±1.7**	49.2±4.9**	60.0±1.2**

$^{++}$ and $^{+}$ denote significant differences between γ-irradiated plants and controls at the 0.01 and 0.05% levels, respectively; ** and * denote significant differences between plants treated with γ-irradiation and plants treated with γ-irradiation+GB at the 0.01 and 0.05% levels, respectively. Data are the means of four replicates ± SE.

increase protein, chlorophyll content and Rubisco activity (Makela et al., 2000). GB treatment improved photosynthesis and enhanced the dry matter yield of barley (Wahid and Shabbir, 2005), and enhanced the CO_2 assimilation and photosynthesis in tobacco (Ma et al., 2007). The present findings were compatible with these reports (Table 1).

Gamma irradiation disturbed the nucleic acid metabolism in fenugreek plants, but GB post-treatment partially reversed the γ-induced detrimental effects (Table 3). The results of the present study revealed enhanced DNase and RNase activities concomitant with a decrease in DNA and RNA levels in fenugreek seeds irradiated with different doses of γ-irradiation (Tables 2 and 3). A similar correlation was reported by Mansour (1994). Machaiah et al. (1976) reported that γ-irradiation blocked the production of the phytohormones that are essential for nucleic acid synthesis.

The role of DNase and RNase in activating the DNA and RNA degradation mechanism induced by γ-irradiation was reported by Bagi et al. (1988) and Danylchenko and Sorochinsky (2005).

The pre-sowing soaking treatment of fenugreek seeds with GB increased the levels of DNA and RNA and proteins (McNeil et al., 1999), which may be attributed to the potential of glycinebetaine to retard the expression of the degradative enzymes, DNase and RNase (Malin et al., 1999). It is suggested that the whole molecular structure of glycinebetaine may be important in the manifestation of radioprotective activity (Monobe et al., 2005). The role of GB is to protect the plant cells against the ravages of γ-irradiation stress by (i) stabilizing the structure of key proteins such as Rubisco (Makela et al., 2000), (ii) increasing photosynthetic activity (Yang et al., 2005), and (iii) functioning as a radical oxygen scavenger (Heuer, 2003).

Table 3

Effect of various doses of γ-irradiation in the presence and absence of GB on the nucleic acids (DNA, RNA) and protein content (mg g FW^{-1}) of fenugreek plants after one month

Parameters	GB (mM)	γ-irradiation doses (Gy)				
		0	25	50	100	150
DNA	0.0	34.5± 1.7	31.5±0.6^{++}	22.4±1.1^{++}	19.3±1.7^{++}	16.8±2.0^{++}
	50	48.6±0.9**	39.5±2.7**	28.6±0.8**	25.9±1.0**	21.4±0.6**
RNA	0.0	80.1±4.0	81.3±3.1	70.1±2.2^{++}	58.4±2.3^{++}	34.6±3.1^{++}
	50	103.5±8.3**	93.4±4.5**	81.2±2.4**	68.5±5.5**	57.3±2.9**
Total protein	0.0	12.7±0.2	11.8±1.2^{+}	9.1±0.2^{++}	7.4±0.1^{++}	5.5±0.1^{++}
	50	16.1±0.6**	13.9±1.2**	12.9±0.3**	10.8±0.3**	9.8±0.2**

$^{++}$ and $^{+}$ denote significant differences between γ-irradiated plants and controls at the 0.01 and 0.05% levels, respectively; ** and * denote significant differences between plants treated with γ-irradiation and plants treated with γ-irradiation+GB at the 0.01 and 0.05% levels, respectively. Data are the means of four replicates ± SE.

Conclusions

This work shows for the first time that glycinebetaine is a potent protector against both the direct and indirect damage caused by radiation. Glycinebetaine has a bipolar structure and contains several chemically reactive methyl groups, which it can donate in enzyme-catalysed reactions (Monobe et al., 2005). GB protection was more significant against irradiation stress at lower doses of γ-rays.

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EFFECT OF SPERMINE AND MINERAL NUTRIENTS ON SUNFLOWER PLANTS GROWN ON A CALCAREOUS SALINE SOIL

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Two field experiments were carried out to investigate the role of seed soaking with spermine (Spm, 10 mg/l) and the foliar application of mineral nutrients (K and Zn), alone or in combination, in improving the tolerance of sunflower (*Helianthus annuus* L.) to calcareous and salinity stress conditions. Both the individual treatments and the interaction increased the stem diameter, shoot fresh and dry weights, yield, yield components and oil yield, as well as the concentrations of K, Ca, P and Zn and the K/Na ratio, whereas they decreased the Na concentration in the two growing seasons. The best results were obtained with the K+Zn+Spm treatment in both seasons.

It could be concluded that seed soaking with Spm and the foliar application of K and Zn might alleviate the harmful effects of calcareous and salinity stress and enhance the ability of sunflower plants to tolerate these adverse conditions.

Key words: sunflower, *Helianthus annuus*, spermine, calcareous and salinity stress, KCl, zinc

Introduction

Sunflower (*Helianthus annuus* L.) represents one of the main oil crops of the world. In Egypt, there is a gap between the production and consumption of plant oils. Increases in the cultivated area of sunflower should be made on reclaimed lands due to the limited land available in the Nile Valley and the competition from major crops.

The presence of $CaCO_3$ directly or indirectly affects the availability of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), iron (Fe), manganese (Mn) and zinc (Zn) (Obreza et al., 1993). On the other hand, salinity has deleterious effects on plant growth, development, seed germination, and the seed characteristics of sunflower, especially oil content. Salinity also influences nutrient uptake. Under these circumstances, potassium and zinc play an

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important role in improving plant development and the subsequent yield of oil crops. Harmati (1993) stated that K application increased the achene and oil yields of sunflower plants on calcareous sandy soil. The foliar application of Zn (60 ppm applied twice, 75 and 85 days after planting) increased the seed yield ha^{-1}, seed index and seed oil content of Egyptian cotton grown on clay loam soil (Sawan et al., 2006), while also improving oil quality.

Polyamines have a specific role in maintaining the cation–anion balance in plant tissues and in stabilizing membranes at high external salinity (Zhao and Qin, 2004). They have an ameliorating effect on all morphological and physiological characters and prevent the degradation of chlorophyll. Polyamines also enhanced the accumulation of organic compounds in a salinity stress study, with the exception of phenols. Therefore, the present investigation aimed to improve the productivity of sunflower plants on calcareous soil with salinity stress using presowing seed soaking with spermine and the application of mineral nutrients (K and Zn) as foliar spray and/or their combinations.

Materials and methods

Two field experiments were conducted on the experimental farm at Maryout Station, Desert Research Center, Egypt during the two growing seasons of 2005 and 2006. The mechanical and chemical analysis of the soil and irrigation water are presented in Table 1. The soil was highly calcareous (31.7% $CaCO_3$), slightly saline (EC 4.7 dS m^{-1}), mildly alkaline (pH 7.9) and loamy in texture. The irrigation water was slightly saline (EC 4.6 dS m^{-1}).

Sunflower seeds were obtained from the Oil Crop Research Institute, Agriculture Research Center, Giza, Egypt. The seeds were sown on June 1st in both seasons.

Table 1
Mechanical and chemical properties of the soil and chemical analysis of the irrigation water at Maryout Research station

Mechanical analysis					
Depth (cm)	Saturation (%)	Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)	Textural class
0–28	41.50	2.06	53.01	21.67	23.26	Sandy clay loam
28–80	43.5	0.74	44.73	24.10	30.43	Clay loam
80–110	47.5	0.40	41.10	34.53	34.53	Clay loam

Chemical analysis												
Depth (cm)	pH	EC (dS m^{-1})	Organic matter (%)	$CaCO_3$ (%)	Cations (meq/L)				Anions (meq/L)			
					Na^+	K^+	Ca^{2+}	Mg^{2+}	CO^{2-}	HCO_3^-	Cl^-	SO_4^{2-}
0.28	7.8	4.30	1.13	31.70	23.90	0.70	8.85	6.32	–	4.37	16.67	17.27
28–80	7.9	3.60	0.28	31.70	21.70	0.60	5.95	3.01	–	1.77	15.50	14.00
80–110	7.9	6.10	–	39.30	34.80	0.60	9.42	6.44	–	1.04	22.50	27.72

Chemical analysis of irrigation water									
pH	EC (dS m^{-1})	Soluble cations (meq/L)				Soluble anions (meq/L)			
		Na^+	K^+	Ca^{2+}	Mg^{2+}	CO_3^{2-}	HCO_3^-	Cl^-	SO_4^{2-}
8.1	4.610	580	27	201	164	–	179.8	783	1050

The seeds were soaked in 10 mg l^{-1} spermine (Spm) for 6 hours before sowing. Unsoaked seeds were used as the control.

Two foliar applications of either potassium as KCl 2.0% and/or zinc as $ZnSO_4$ 0.01% were given, the first 5 weeks after sowing and the second after a one-week interval. Distilled water was used as the control. Tween 20 was used as wetting agent at 0.05%. Automatic atomizers were used.

The treatments were arranged in a complete block design with three replicates. The experimental unit measured 9 m^2 (3 × 3 m) with row and plant spacings of 30 cm. The treatments were as follows: 1. Control, 2. Spermine (Spm) 3. Potassium, KCl 2% (K), 4. Zinc, $ZnSO_4$ 0.01% (Zn), 5. K + Zn, 6. K + Spm, 7. Zn + Spm, 8. K + Zn + Spm.

The recommended fertilization for this type of soil was applied according to the Desert Research Center as follows: Organic fertilization (47.6 m^3 per hectare) during soil preparation, $Ca(H_2PO_4)_2$ (107 kg P_2O_5/ha) before sowing, and NH_4NO_3 and K_2SO_4 (119 kg/ha each, 3 times, at 2, 4 and 6 weeks after sowing).

Two samples were taken, the first at 50 days after sowing for the determination of growth parameters (plant height, stem diameter at the 10th node from the top, leaf number, plant fresh and dry weights) and for chemical analysis (Na, K, Ca, P and Zn) from the leaves at the 7th node from the top. Crude dried leaf material was digested using the wet ashing procedure as described by Johanson and Ulrich (1959) and the contents of Na, K and Ca were determined using a Jenway PFP7 flame photometer (Brown and Lilleland, 1946). Zn and Mn were estimated using an atomic absorption spectrophotometer (Pye Unicium Spmm 1900) and phosphorus (P) according to Murphy and Riley (1962).

The second sample was taken at harvest (90 days after sowing) to determine yield components, including head diameter, seed number/head, seed weight/head, 100-seed weight and seed yield (t/ha). Chemical analysis was conducted on the seeds to determine the oil percentage (A.O.A.C., 2000).

The data from all the experiments were subjected to statistical analysis of variance. The differences between the means of the studied traits were judged by Duncan's multiple range test according to Gomez and Gomez (1984).

Results and discussion

Growth parameters

The data in Table 2 show that the foliar application of K or Zn under salt soil stress increased the plant height, stem diameter, shoot fresh and dry weights of sunflower in both growing seasons. Moreover, seed soaking with Spm increased the stem diameter and shoot fresh and dry weights of sunflower plants.

All the combined treatments increased the growth parameters of sunflower plants except for leaf number in both growing seasons. The K+Zn+Spm treatment was the most effective in enhancing the growth parameters and counteracting the harmful effects of calcareous saline soil, followed by Zn+Spm.

The inhibitory effect of soil salt stress on sunflower growth may be due to a decrease in water absorption, metabolic processes, meristematic activity and/or cell enlargement (Khadr et al., 1994; Sakr et al., 2008) or to damage to the cells, so that they cannot perform their functions (Chen and Murata, 2002).

The reason for the promotive effect of K on sunflower growth could be that K increases the photosynthetic rate and CO_2 assimilation and has an important role in the translocation of photosynthates from source to sink (Sangakkara et al., 2000). Furthermore, K plays an important role in osmotic adjustment under saline conditions to maintain the selectivity and integrity of cell membranes (Satti and Lopez, 1994).

Table 2
Effect of foliar application of nutrient minerals (K and Zn) and seed soaking with spermine (Spm) and their combinations on the growth parameters of sunflower plants at 50 days after planting in two growing seasons (2005 and 2006)

Treatments	Plant height (cm)	Stem diameter (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	No. of leaves/plant	Plant height (cm)	Stem diameter (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	No. of leaves/plant
	2005					2006				
Control	122^{a}	1.8^{a}	683^{a}	76^{a}	23^{a}	124^{a}	2.0^{a}	742^{a}	93^{a}	22^{a}
K	125^{b}	2.2^{c}	932^{d}	105^{e}	23^{a}	130^{b}	2.5^{b}	914^{c}	112^{b}	22^{a}
Zn	127^{bc}	2.0^{b}	794^{b}	87^{b}	23^{a}	133^{b}	2.5^{b}	885^{b}	109^{b}	22^{a}
Spm	120^{a}	2.0^{b}	841^{c}	93^{c}	22^{a}	124^{a}	2.5^{b}	929^{d}	114^{b}	22^{a}
K+Zn	126^{bc}	2.2^{c}	850^{c}	95^{c}	23^{a}	135^{c}	2.5^{b}	890^{b}	110^{b}	22^{a}
K+Spm	125^{b}	2.0^{b}	840^{c}	93^{c}	23^{a}	130^{b}	2.0^{a}	930^{d}	114^{b}	22^{a}
Zn+Spm	125^{b}	2.2^{c}	930^{d}	101^{d}	23^{a}	124^{a}	2.8^{c}	1017^{e}	127^{c}	22^{a}
Zn+K+Spm	129^{d}	2.3^{c}	940^{d}	110^{e}	23^{a}	132^{b}	2.9^{c}	935^{d}	130^{c}	22^{a}

Values within the same column having the same letter are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

Zinc performs various important roles in protecting cells from the damage caused by reactive oxygen species (ROS) in plants grown under salt soil stress. Zinc is particularly needed within the environment of plasma membranes to maintain their structural and functional integrity (Cakmak, 2001). Zn-deficiency-related disturbances in the cellular metabolism are responsible for oxidative damage to membrane proteins, phospholipids, chlorophyll, nucleic acids, SH-containing enzymes and IAA, thus inhibiting plant growth. Recent reports demonstrate that the shoot and root meristematic activities of plants are rapidly blocked under oxidative stress conditions as a result of DNA damage (Reicheld et al., 1999). Very high concentrations of Zn in meristematic plant cells (Kitagishi and Obata, 1986; Hossain et al., 1997) demonstrate the crucial roles played by Zn in highly metabolically active differentiating cells.

Many investigators have reported the effect of polyamines (PAs). It has been suggested that PAs may play a role in the antioxidative system and protect membranes from peroxidation. The alleviating effect of polyamines on plants grown under salinity stress may be due to a number of factors, including the activation of the antioxidative defence system (Chattopahyay et al., 2002), the suppression of superoxide and H_2O_2 levels (Hernandez et al., 1995) thus reducing membrane damage, a reduction in ROS through the quenching of singlet oxygen and excited chlorophyll by elevating the level of carotenoids, thereby maintaining the chloroplastic membrane (Velikova et al., 2000), a reduction in membrane leakage and lipid peroxidation and a decrease in the monodehydroascorbate (MDA) content, as observed in sugarcane leaves (Zhang

and Kirkham, 1996), the stabilization of the membranes and an increase in ascorbic peroxidase (AXP) and glutathione reductase (GR) activity (Tiburcio et al., 1994), the stimulation of chlorophyll synthesis and the prevention of chlorophyll degradation (Krishnamurthy, 1991).

It could be concluded that the mineral nutrients K and Zn alleviated the harmful effect of calcareous-saline soil on sunflower plants. The application of antioxidants (spermine) also proved to be effective in this respect.

Yield and yield components

The data in Table 3 showed that all the foliar applications of mineral nutrients increased the head diameter, number of seeds (achenes)/head, seed weight/head, 100-seed weight and seed yield during the 2005 and 2006 seasons. Zn treatment was more effective than K in this respect. Seed soaking in Spm significantly enhanced seed number/head, seed weight/head, 100-seed weight and seed yield in both seasons.

Head diameter, number of seeds per head, seed number/head, seed weight/head and seed yield were significantly increased by the combined treatments during the two seasons. The highest values of seed number, seed weight/head and seed yield were recorded after the application of K+Zn+Spm, in comparison with control plants. It could be concluded that the K+Zn+Spm treatment was the most effective in alleviating the harmful effects of calcareous and salinity stress on yield and its components in sunflower plants.

Table 3

Effect of foliar application of nutrient minerals (K and Zn) and seed soaking with spermine (Spm) and their combinations on the yield and yield components of sunflower plants at 90 days after planting in two growing seasons (2005 and 2006)

Treatments	Head diameter (cm)	No. of seeds/head	Seed weight /head (g)	100-seed weight (g)	Seed yield (kg/ha)	Head diameter (cm)	No. of seeds/head	Seed weight /head (g)	100-seed weight (g)	Seed yield (kg/ha)
	2005					2006				
Control	19.0^{a}	867^{a}	86^{a}	7.2^{a}	2277^{a}	21.8^{a}	1010^{a}	85^{a}	6.1^{a}	2258^{a}
K	21.0^{b}	1191^{b}	108^{b}	9.2^{c}	3729^{b}	23.5^{c}	1381^{b}	106^{b}	8.3^{bc}	3679^{b}
Zn	21.3^{b}	1219^{c}	115^{c}	9.3^{c}	3993^{b}	23.5^{c}	1404^{c}	113^{c}	8.8^{bc}	3905^{c}
Spm	20.1^{a}	1201^{c}	107^{b}	8.7^{b}	3831^{b}	23.9^{c}	1382^{b}	104^{b}	7.9^{b}	3617^{b}
K+Zn	20.2^{ab}	1220^{b}	120^{c}	9.2^{c}	3998^{b}	23.5^{c}	1410^{c}	115^{c}	8.9^{bc}	3927^{c}
K+Spm	20.6^{b}	1170^{b}	111^{b}	9.3^{c}	3831^{b}	23.9^{c}	1374^{b}	108^{b}	8.2^{bc}	3705^{b}
Zn+Spm	19.9^{a}	1447^{d}	129^{d}	9.6^{c}	4479^{c}	22.8b^{c}	1550^{d}	125^{d}	9.0^{c}	4333^{d}
Zn+K+Spm	21.9^{b}	1460^{d}	135^{e}	9.9^{d}	4522^{c}	24.1^{d}	1560^{d}	130^{d}	9.8^{d}	4379^{d}

Values within the same column having the same letter are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

It has been stated by other authors that the reduction in seed yield by soil salt stress is largely due to a reduction in pollen viability, which is important in pollen germination and pollen tube growth, the abscission of flowers or young fruit due to ethylene induction by salinity, decreases in pollen grain production, mean number of perfect flowers and fruit set, and a decline in the leaf area and number per plant, resulting in a reduced supply of carbon assimilates due to lower net photosynthetic rate and biomass accumulation (Sakr et al., 2008).

These results confirmed the findings of Mekki et al. (1999) on sunflower plants. As K is required as a cofactor for many enzymes involved in respiration and photosynthesis, K application could result in a gain in carbon fixation and energy production, with a positive effect on oil seed yield.

Plants supplied with Zn showed a significant increase in head diameter, number of seeds, seed weight/head, 100-seed weight and seed yield during the 2005 and 2006 seasons. These results were in agreement with the findings of Sawan et al. (2006) on the seed yield of Egyptian cotton.

In wheat plants, decreases in grain yield due to salinity stress were more marked in Zn-deficient plants (Ekiz et al., 1998). By affecting the synthesis and activity of antioxidative enzymes, Zn is an important factor against destructive O_2 species in plant defence systems. Thus, an improvement in the Zn nutritional status of plants may be of great importance for their survival under oxidative stress conditions such as drought, chilling, high light levels, ozone and salinity (Cakmak, 2001).

The efficiency of Zn in improving the productivity of sunflower may be due to its ability to increase the auxin level and to promote the hormonal balance within the plant tissues and the condensation of amino acids into protein (Jefferey, 1987).

As for the role of exogenous antioxidants on alleviating salinity stress effects, PAs such as spermine (Spm, a tetramine), spermidine (Spd, a triamine) and their obligate precursor putrescine (a diamine) are implicated in the induction of plant adaptation to stresses (Mishra et al., 2003). It has been suggested that PAs may play a role in the antioxidant system and protect membranes from peroxidation.

The antioxidants applied in the present work were able to alleviate or minimize the harmful effect of NaCl salinity on sunflower growth.

Biochemical constituents

The data presented in Table 4 show that the foliar application of K and Zn or seed presoaking in spermine significantly decreased the Na content and increased K, K/Na, Ca, P and Zn during the two seasons. The combined treatments reduced Na content, for which higher values were recorded in control plants grown on calcareous saline soil in the two seasons. The application of K+Zn+Spm gave the best results in terms of the lowest values of Na and the highest values of K, K/Na, Zn and P in the two growing seasons.

Table 4
Effect of foliar application of nutrient minerals (K and Zn) and seed soaking with spermine (Spm) and their combinations on the oil % and oil yield (kg/ha) of sunflower plants at 50 days after planting in two growing seasons (2005 and 2006)

Treatments	Oil (%)	Oil yield (kg/ha)	Oil (%)	Oil yield (kg/ha)
	2005		2006	
Control	34.3[a]	971[a]	34.7[b]	975[a]
K	35.1[a]	1306[bc]	35.8[b]	1318[b]
Zn	37.7[bc]	1549[d]	37.9[c]	1561[c]
Spm	36.4[b]	1368[c]	36.2[c]	1347[b]
K+Zn	36.0[bc]	1380[c]	35.3[bc]	1368[b]
K+Spm	36.6[c]	1287[b]	36.0[c]	1299[b]
Zn+Spm	38.0[d]	1594[e]	32.0[a]	1606[d]
Zn+K+Spm	38.2[d]	1606[e]	38.0[d]	1611[d]

Values within the same column having the same letter are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

In a saline environment, plants take up excessive amounts of Na^+ and Cl^-, resulting in high Na^+/Ca^{2+} and Na^+/K^+ ratios, which may impair the selectivity of the root membrane (Khan et al., 1997). The greater accumulation of Na^+ in plant roots may be due to a regulatory mechanism located within the roots that prevents the translocation of excessive cations, such as Na^+, from the root to aerial parts, resulting in Na^+ retention.

Chloride is a more sensitive indicator of salt damage than sodium, since it is stored by the plant. The accumulation of Cl^- may cause leaf injury, thereby decreasing photosynthesis and productivity. Ahmed et al. (2002) proposed that the relatively greater uptake of Cl^- than Na^+ in salt-stressed plants could be responsible for growth reduction by depressing the uptake of other anions.

The reduction of internal potassium concentration could be related to an increase in potassium efflux into the growth medium (Cramer et al., 1989), the disruption of membrane integrity by Na^+ and the inhibited transport of this ion into the roots and up to the shoots (Wu et al., 1999), the antagonism between K^+ and Na^+ cations, which increased considerably as salinity increased (Sairam and Srivastava, 2002), or the passive accumulation of Na^+ in the roots and shoots leading to a high Na^+/K^+ ratio and reduced plant growth.

It was found that the application of K and trace elements increased the P concentration in soybean, which could be attributed to the role of K in osmotic adjustment and in maintaining the selectivity and integrity of cell membranes (Satti and Lopez, 1994).

It appears that trace elements play a role in the regulation of ion uptake in sunflower grown under salinity. Trace elements may also modify the movement of nutrients within the plant, causing changes in nutritional requirements under salinity (Hatung, 2004).

The decrease in P concentration associated with salinity conditions may be ascribed to high pH values, which might hinder P availability to the plants (Greenway and Munns, 1980) or to a decrease in the translocation of P upward through the stem because of an increase in the osmotic pressure of the root medium (Sakr et al., 2008).

The decrease in calcium concentration under salinity may be due to the reduced activity of calcium in the solution at high sodium levels, a decrease in the amount of calcium available for uptake by the plant, the antagonism between sodium and calcium at the site of uptake in the roots, or to inhibition of uptake processes.

In the present investigation, better growth and yield were associated with lower Na^+ content, higher K content and a higher K/Na ratio as a result of Spm treatment. In this context, many investigators reported that Spm has a diminishing effect on Na concentration and has a promotive effect on K, P and Zn (Tiburcio et al., 1994; Velikova et al., 2000; Chattopadhyay et al., 2002).

It could be concluded that the application of exogenous plant antioxidants could alleviate the harmful effects of salinity stress on the biochemical constituents of wheat plants.

Oil percentage and oil yield

The data in Table 5 clearly demonstrate that the foliar application of K and Zn or seed presoaking in spermine significantly increased the oil percentage and oil yield (kg/hectare) of sunflower seeds in both seasons. The application of Zn resulted in the highest mean values of oil % and oil yield compared with control plants in both seasons.

Table 5

Effect of foliar application of nutrient minerals (K and Zn) and seed soaking with spermine (Spm) and their combinations on the mineral element concentrations of sunflower plants at 50 days after planting in two growing seasons (2005 and 2006)

Treatments	Na (mg/g DW)	K (mg/g DW)	Ca (mg/g DW)	K/Na ratio	P (mg/g DW)	Zn mg/100 g DW	Na (mg/g DW)	K (mg/g DW)	Ca (mg/g DW)	K/Na ratio	P (mg/g DW)	Zn mg/100 g DW
	2005						2006					
Control	18.4^{c}	44.4^{a}	79.0^{a}	2.41^{a}	0.28^{a}	1.16^{a}	15.8^{d}	46.5^{a}	66.6^{a}	2.94^{a}	0.22^{a}	0.91^{a}
K	16.1^{b}	47.1^{b}	82.9^{b}	2.93^{b}	0.40^{c}	1.55^{b}	13.9^{bc}	54.9^{b}	69.0^{c}	3.94^{b}	0.40^{c}	1.10^{b}
Zn	16.4^{b}	46.9^{b}	81.3^{b}	2.86^{b}	0.36^{b}	1.54^{b}	14.4^{c}	52.2^{b}	67.5^{b}	3.62^{b}	0.33^{b}	1.10^{b}
Spm	15.9^{ab}	47.3^{b}	89.3^{c}	2.97^{b}	0.40^{c}	1.65^{b}	13.9^{b}	53.2^{b}	71.0^{cd}	3.76^{b}	0.40^{c}	1.30^{c}
K+Zn	16.5^{b}	48.0^{c}	83.0^{b}	2.90^{b}	0.40^{c}	1.60^{b}	14.5^{c}	55.0^{b}	70.0^{c}	3.80^{b}	0.40^{c}	1.10^{b}
K+Spm	16.0^{b}	47.0^{b}	90.0^{c}	2.94^{b}	0.40^{c}	1.65^{b}	13.7^{bc}	53.0^{b}	70.0^{c}	3.89^{b}	0.30^{b}	0.90^{a}
Zn+Spm	14.0^{a}	49.0^{cd}	86.0^{d}	3.50^{c}	0.40^{c}	1.80^{c}	12.8^{a}	60.0^{c}	70.0^{c}	4.69^{c}	0.52^{d}	0.95^{a}
Zn+K+Spm	13.5^{a}	50.0^{de}	92.0^{e}	3.70^{c}	0.45^{d}	1.85^{c}	12.0^{a}	63.0^{c}	72.0^{e}	5.25^{d}	0.55^{d}	1.35^{c}

Values within the same column having the same letter are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

The combined treatments K+Zn, K+SP, Zn+SP and K+Zn+SP increased the values of oil % and oil yield (kg/hectare) compared with untreated plants in both seasons. Zn acted synergistically with Spm, showing higher values compared with the joint application of Zn and K. The K+Zn+Sp treatment gave the best results in the two growing seasons.

The enhancing effect of Zn, K and Spm on growth parameters, the accumulation of dry mass and the biochemical constituents of sunflower plants may be reflected in the increasing oil yield.

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INFLUENCE OF NITROGEN AND HERBICIDE TREATMENTS ON THE NITROGEN UPTAKE OF PEA AND *Chenopodium album* L.

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Based on the results of earlier greenhouse tests, a field experiment was conducted to evaluate the effects of three different herbicide combinations (clomazone, flumioxazine and pendimethaline combined with bentazone) at increasing nitrogen levels on the nitrogen uptake of green pea and common lambsquarters (*Chenopodium album* L.). Nitrogen was administered to the pea plants in the form of ammonium nitrate at increasing levels: 0, 100, 200 and 300 kg/ha. The experimental soil was loamy Ramann's brown forest soil (Eutric Cambisol). Green peas were grown to green maturity and harvested according to standard agricultural practices. Following harvest, the fresh and dry weight of the pea and weed shoots were recorded. The nitrogen, phosphorus and potassium contents were determined from dried plant samples after digestion with concentrated sulphuric acid.

The main results can be summarized as follows:

The addition of nitrogen to the treatments considerably altered the growing potential of pea plants, especially at the early growth stage, where an increase in dry biomass of nearly 30% was observed. Yield biomass decreased in the nitrogen treatments. The nitrogen concentration, which increased in every treatment, was directly correlated to the addition of nitrogen fertilizer. Herbicides mainly influenced the vegetative growth of the plants. Pendimethalin and flumioxazin indirectly caused an increase in the dry biomass of the shoots by killing the surrounding weeds.

Key words: *Pisum sativum*, *Chenopodium album*, herbicide treatments, dry weight, nitrogen %

Introduction

Field peas are an important component of feed mixtures and also form an important forage crop alone, which is sown in autumn and spring. One advantage of using peas in the crop rotation is that the soil has a higher N content and is better conditioned due to microbiotic nitrogen fixation. Green peas have a short growing period which can be followed by a second crop. Both

fresh and dry peas contain carbohydrates and protein in a good ratio for human consumption. In addition, the fibre content is one of the highest of all vegetables and the crop also contains a considerable amount of vitamin C (Salunkhe and Kadam, 1998).

The pea plant has a high nutrient demand, since it has a shallow root system and a relatively short growing period. The crop has a particularly high phosphorus and potassium requirement. Although a nitrogen-accumulating plant, it still needs nitrogen in spring, since the Rhizobium bacteria only absorb nitrogen intensively 4–5 weeks after the plants emerge. However, if the soil nitrogen supply is excessive, the Rhizobium bacteria will fail to absorb nitrogen and the plants will use the nitrogen supply from the soil itself (Jensen, 1986; Eskin, 1989; Izmailov and Ovcharenko, 1995). Nitrogen and potassium uptake takes place mainly up to the end of flowering, while phosphorus uptake is intensive until the end of the grain-filling period (Kismányoky, 2005).

Pea is extremely sensitive to weed competition, so great attention must be paid to its cultivation. Herbicides with different modes of biological action may have different effects on the life processes of the pea plant. Herbicides may hinder pea development or cause direct damage. The plant may compensate for these effects later on, but may also suffer lasting damage leading to yield reduction.

Many herbicides affect nitrogen sources in the soil, killing part of the microbe population and creating problems in the transformation of nitrogen forms in the soil. Some herbicides are known to inhibit various processes such as nitrification, denitrification and nitrogen fixation. Based on previous research (Szentpétery et al., 2005; Jolánkai et al., 2006; Wágner and Nádasy, 2007) some herbicides influence nutrient uptake and yield quality.

Dhima and Eleftherohorinos (2005) investigated the effect of nitrogen supply on interspecific competition between wild mustard (*Sinapis arvensis* L.) and wheat, barley and triticale. The presence of wild mustard reduced the dry weight of wheat and triticale by 31 and 26%, respectively, by harvest, while the corresponding reduction for barley was only 1.5%. The grain yield of wheat and triticale was also reduced. In addition, the presence of wild mustard reduced the total N content of wheat and triticale by 20%.

Wall et al. (1991) and Wall and Townley Smith (1996) determined the competitive effect of wild mustard on two field pea cultivars. For both cultivars, 20 wild mustard plants/m^2 reduced yields by 2–35%.

The N and P percentages in spring barley did not change under the influence of increasing levels of nitrogen and phosphorus when they grew in competition with weeds (Andreasen et al., 2006). *Sinapis arvensis* had a larger uptake of phosphorus than spring barley, in spite of the fact that the dry weight of spring barley was considerably larger.

Varga et al. (2000) examined the competition between maize and barnyardgrass (*Echinochloa crus-galli* L.), and established that 26 weed plants/m^2 reduced seed yields by 44.77% compared with the herbicide-treated control.

According to Kádár et al. (2003), at nitrogen doses of above 100 kg ha^{-1} the pea cover decreased and the weed cover increased. When the phosphorus supply was improved, both the pea and weed cover increased.

The effect of mineral nitrogen availability on nitrogen nutrition and biomass was investigated by Voisin et al. (2002) under adequately watered conditions in the field, using five levels of fertilizer nitrogen application at sowing (0, 50, 100, 200 and 400 kg N/ha). The excessive nitrogen and biomass accumulation in the shoots of the 400 kg N treatment caused crop lodging and slightly depressed the seed yield and seed nitrogen content.

Singh and Wright (2002) studied the effects of three herbicides on the node number, growing patterns and yield of two pea cultivars under greenhouse conditions. Double doses of Basagran (active ingredient: bentazone) caused chlorosis of the plants. The number of nodes was reduced, particularly by double doses of Basagran and Gesagard, and all the herbicides decreased the dry matter of the shoots and peas.

Al Khatib and Tamhane (1999) used low-concentration sulfonylurea products pre- and post-emergence to control weeds in pea. The effects of different adjuvants on bentazone efficacy were investigated by Al Khatib et al. (1995).

The aim of the present field experiment was to study the reactions of pea plants and one important weed, common lambsquarters [*Chenopodium album* (L.)] to the applied herbicides and the various nitrogen treatments.

Materials and methods

Based on the results of earlier greenhouse tests, a field experiment was conducted to evaluate the effects of three different herbicides at increasing nitrogen levels on the nitrogen uptake of green peas and the important weed, common lambsquarters (*Chenopodium album* L.). Field trials were conducted in Keszthely, Hungary, in the spring of 2007. A randomized complete block design with three replications was used in the experiment.

The herbicide combinations applied in the experiment were clomazone + bentazone, flumioxazin + bentazone and pendimethaline + bentazone.

The green pea variety Ambassador was sown in 12 cm rows on 27th March 2007. The individual plot size was 5 × 5 m^2. Nitrogen (N_0 = 0, N_1 = 100, N_2 = 200 and N_3 = 300 kg/ha) was incorporated one week before sowing. The pre-emergence herbicides were applied within 2 days of seeding using a J-14 type knapsack sprayer with a spray volume of 500 L/ha. The post-emergence herbicide was sprayed three weeks after sowing. The treatment consisted of three doses of nitrogen fertilizers and a control, and three herbicide combinations and a control (Table 1), making a total of 16 treatments.

The experimental soil was loamy Ramann's brown forest soil (Eutric Cambisol) with the following main characteristics: humus content 2.05% (medium supplies); upper limit of plasticity according to Arany (K_A): 39; pH (H_2O): 7.26; pH (KCl): 6.48; mineral N content: 24.08 mg/kg; AL-P_2O_5 content: 241.19 mg/kg (very good supplies); AL-K_2O content: 244.24 mg/kg (good

supplies). Only the nutrients in the soil were available to the plants (except for the small quantity of atmospheric nitrogen fixed by pea), as no fertilizer was applied. A fungicide/insecticide mixture was applied once to control fungal diseases and insect pests. The peas were grown to green maturity and harvested according to standard agricultural practices. Samples were collected on two different dates. The first sample was taken at the 2–3-leaf stage (BBCH 12–13) and the second at green maturity (BBCH 79). At the second sampling date *Chenopodium album* samples were collected at the BBCH 65 stage.

The data recorded were crop dry weight, dry yield biomass (pods and grains), and the nitrogen content of green pea and *Chenopodium album* (L.). All the data were subjected to analysis of variance (ANOVA) and analysed using SPSS 9.0 for Windows.

Table 1
Characteristics of the herbicides applied in the experiment

Active ingredients	Rate	Mode of application
Clomazone	96 g a. i./ha	Pre-emergence
Flumioxazine	40 g a. i./ha	Pre-emergence
Pendimethaline	1320 g a. i./ha	Pre-emergence
Bentazone	1440 g a. i./ha	Post-emergence

Results

Dry weight of pea shoots

Major differences were observed between the dry weight production of the untreated control and the nitrogen-treated pea plants. Compared with the nitrogen control, the dry shoot weight of the peas on the 100 kg/ha N plots was found to increase by 20% (Fig. 1). At the 200 kg/ha N level the dry biomass continued to increase, but at the 300 kg/ha level there was a drop in the dry biomass (Fig. 1). Although numerical differences were observed at the end of the vegetation season (Fig. 2) in response to nitrogen treatment, there were no significant differences.

The herbicide treatments had a greater influence on shoot growth at the higher nutritional levels. At the start of the growing season the herbicides reduced the dry weight of the pea shoots, while at the end of the vegetation season they had little effect. Compared to the herbicide control (untreated plots) the dry weight of the pea shoots was increased by pendimethalin and clomazone with bentazone and decreased by flumioxazin. Once green pod ripening had commenced, all the treatments were similar except clomazone-treated plants, which showed significant shoot growth.

Dry yield biomass

The dry yield biomass decreased from 41 to 37 g as the nitrogen level rose. In some instances, herbicides increased the yield biomass in comparison with the herbicide controls. Interestingly, pendimethalin caused an increase at the 100 and 200 kg/ha nitrogen levels (Fig. 3).

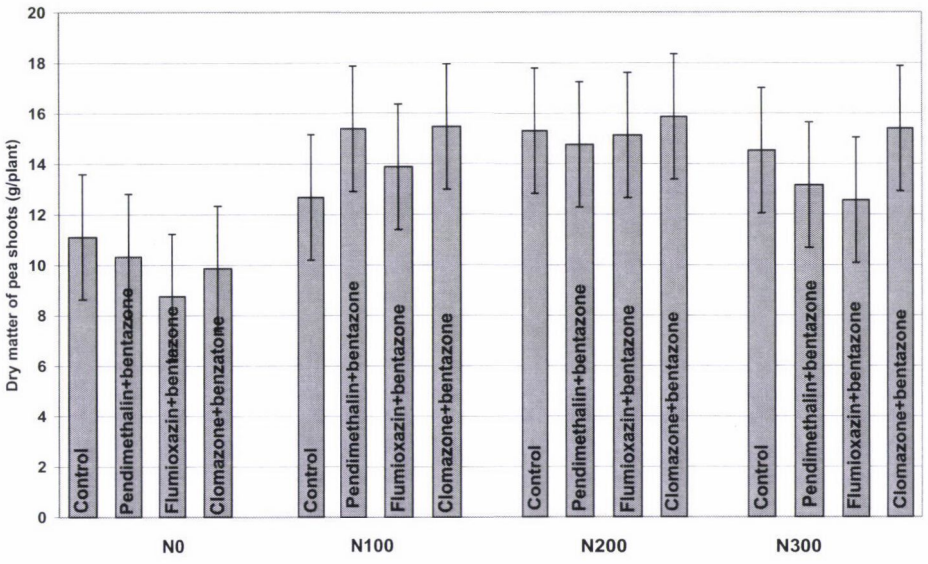

Fig. 1. Dry weight of pea shoots at the 2–3-leaf stage (BBCH 12-13)

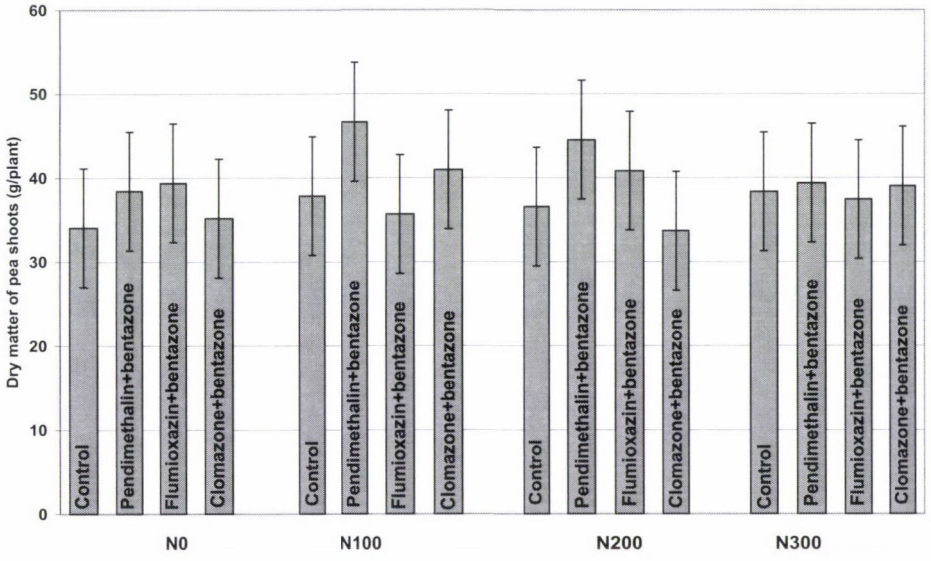

Fig. 2. Dry weight of pea shoots at green maturity (BBCH 79)

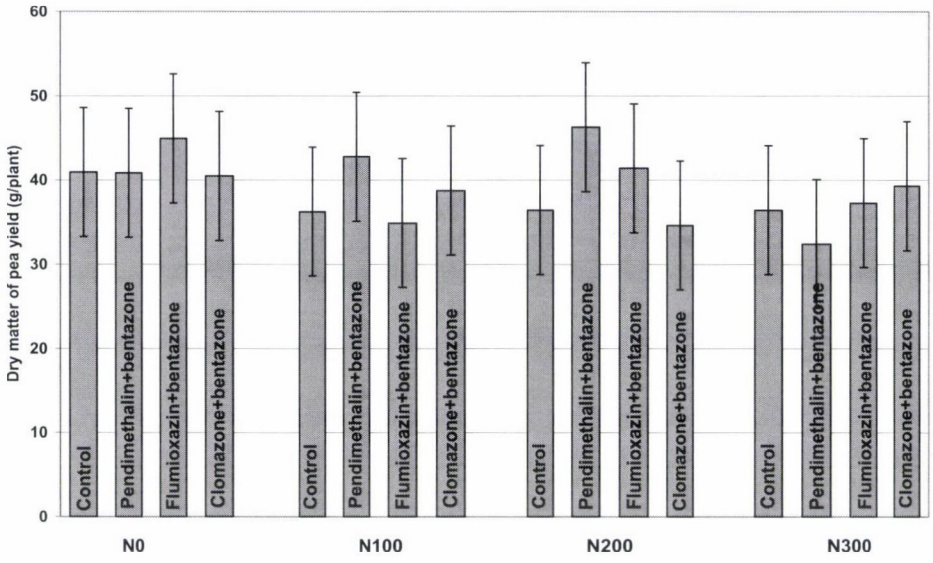

Fig. 3. Dry yield biomass of pea

Dry weight of Chenopodium album *L.*

An increase in nitrogen fertilizer was directly correlated to an increase in dry matter production in the treated plots compared to the control (no fertilizer or herbicide), with an increase of 56% at the vegetative stage, 64% at flowering and 61% at green maturity (Fig. 4). Clomazone-treated plants showed a significant decrease in dry matter accumulation at the 100 and 300 kg/ha nitrogen levels. This herbicide caused definite symptoms on the weeds: yellowing leaf spots were first seen around the borders of the leaves, and eventually the leaf turned completely white.

Nitrogen uptake of pea and Chenopodium album *L.*

The nitrogen content of the shoots rose proportionally to the nitrogen fertilizer rate, from 3.97% to 4.75% after germination and from 1.6% to 2.5% at ripening. At the 2–3-leaf stage the herbicides had no effect on this parameter, but at green maturity clomazone and pendimethalin caused a significant increase and flumioxazin a slight reduction (Table 2).

The nitrogen percentage in the pods, like that of the shoots, increased after the application of nitrogen, reaching a maximum at the 100 kg/ha nitrogen level. Herbicides caused a further 1% increase.

The nitrogen concentration of *Chenopodium album* shoots was 3% in the absolute control, increasing significantly to 3.8% in the N100 herbicide control. After stagnating in the N200 treatment it increased to 4.6% at the next nitrogen level. The herbicides had little influence on weed nitrogen uptake, while the nitrogen treatment had a significant effect.

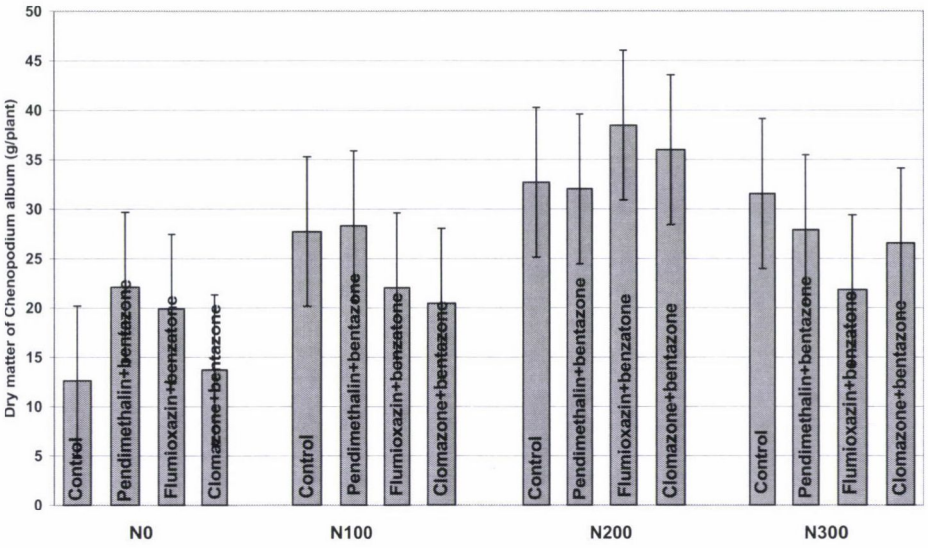

Fig. 4. Dry weight of *Chenopodium album* L.

Table 2
Nitrogen content of pea shoots, pea yield and *Chenopodium album* L.

Treatments	N content (%) of pea shoots at BBCH 12-13	N content (%) of pea shoots at BBCH 79	N content (%) of pea yield	N content (%) of CHEAL shoots
N0				
Control	3.973	1.669	3.960	3.000
Pendimethalin+bentazone	3.950	1.832	4.400	3.626
Flumioxazin+benzatone	3.833	1.659	4.240	3.786
Clomazone+bentazone	3.943	1.891	4.410	3.103
$LSD_{5\%}$	0.171	0.166	0.164	0.261
N100				
Control	4.267	2.059	4.510	3.694
Pendimethalin+bentazone	4.490	2.046	4.730	3.301
Flumioxazin+benzatone	4.200	1.784	4.710	3.583
Clomazone+bentazone	4.220	1.925	4.130	3.419
$LSD_{5\%}$	0.183	0.172	0.168	0.265
N200				
Control	4.847	2.350	4.163	3.693
Pendimethalin+bentazone	4.657	2.467	4.300	4.026
Flumioxazin+benzatone	4.737	2.164	4.270	4.160
Clomazone+bentazone	4.577	2.100	4.687	4.226
$LSD_{5\%}$	0.189	0.173	0.165	0.259
N300				
Control	4.757	2.546	4.133	4.551
Pendimethalin+bentazone	4.927	2.900	4.123	4.629
Flumioxazin+benzatone	5.047	2.457	4.053	4.667
Clomazone+bentazone	4.853	3.085	3.980	4.608
$LSD_{5\%}$	0.191	0.178	0.167	0.274

Discussion

Effect of nitrogen fertilizer

It could be seen from the results that nitrogen treatment caused a considerable alteration in plant growth, especially at the early growth stage, when pea dry weight increased by nearly 30%. At the highest nitrogen level of 300 kg/ha there was a slight decrease in pea dry weight, possibly due to the height of the weeds, which shaded the crop, increasing competition between the two species. The high soil nitrogen concentration originated from the high level of nitrogen fertilization, which was used with greater efficiency by common lambsquarters than by pea. This weed species is a typical nitrophilous plant, thriving in a habitat rich in nitrogen and able to utilize extremely high soil nitrogen content, producing greater dry biomass. High nitrogen levels may cause nitrogen–phosphorus and nitrogen–potassium antagonism, in which case nitrogen may hinder the uptake of phosphorus and potassium ions.

As in an earlier study (Wágner and Nádasy, 2009) the yield biomass was decreased by nitrogen treatment. The plants require mainly phosphorus and potassium for the development of the pods, but the high nitrogen levels decreased the phosphorus and potassium content of the soil, reducing their availability to the crops.

Nitrogen application increased the nitrogen concentration in peas in all the treatments. There was a positive correlation between the nitrogen content of the pods and the nitrogen dose.

Effect of herbicides

Herbicides had the greatest influence on the vegetative organs. Pendimethalin and flumioxazine combined with bentazone increased the dry matter of the shoots indirectly by killing the weeds, thus eliminating competition for the available resources. Because of this the peas obtained more water, nutrients and living space, allowing them to grow larger.

Flumioxazine was the herbicide active ingredient that caused the greatest decrease in dry biomass. The vegetative growth of *Chenopodium album* was also reduced by the combination of clomazone and bentazone.

Bentazone is known to decrease the specific nitrogenase activity in kidney beans (Schnelle and Hensley, 1990) and soybean (Ozair and Moshier, 1988). The lower nodulation and nitrogenase activity in herbicide-treated plants could be due to poor shoot growth as the result of decreased photosynthesis. This was also observed for the herbicides used by Singh and Wright (2006), which similarly had an adverse effect on photosynthesis. This could be why the shoot nitrogen content was reduced by the combination of flumioxazin+bentazone at green maturity.

Conclusions

This study indicated that nitrogen fertilizer applied at concentrations of 100 or 200 kg/ha was utilized by pea plants for vegetative growth and development. The application of 300 kg/ha of nitrogen was less beneficial due to nutrient competition with the weeds. Although nitrogen fertilizer caused a slight reduction in yield, the nitrogen and protein contents of the pods did not decrease.

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SEED GERMINATION AND STORAGE RESERVES OF MAIZE AND SORGHUM AFTER EXPOSURE TO AND RECOVERY FROM PRE- AND POST-FLOWERING DEHYDRATION

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Investigations were made on the seed viability (standard germination test and vigour after accelerated ageing) and seed quality (starch, protein, lipid, sucrose, glucose and fructose) of seeds of maize (cv Melkassa-2) and sorghum (cv Macia) harvested from plants after exposure to and recovery from pre- and post-flowering dehydration. The objectives of the study were to achieve a better understanding of 1) the effects of water deficit during the pre- and post-flowering stages on the seed viability and food (storage reserves) quality, and 2) the effects of dehydration and rehydration cycles at critical growth stages on subsequent seed performance and production, which could lead to the development of cultivars more able to efficiently partition assimilates to the reproductive organs in the field. The experiment was conducted in a controlled environment growth chamber under constant environmental conditions (12/12 h day/night, 28–32/17°C day/night temperature, 60–80% RH and 1200–1400 μmol m^{-2} s^{-1} PPDF). The seed viability and vigour tests were done on air-dried seeds and the seed quality analysis on freeze-dried seeds of both species.

The results of the standard germination test indicated that sorghum seeds harvested after both pre- and post-flowering dehydration were not affected by the treatment, while maize seeds had reduced % germination. Sorghum seeds harvested after post-flowering dehydration had significantly decreased vigour after accelerated ageing. Dehydration during both the pre- and post-flowering stages resulted in reduced contents of protein, lipid and soluble carbohydrates (sucrose, glucose and fructose) in both species as compared to the control seeds. The species differed in the extent to which these reserves were reduced.

Key words: dehydration, seed viability, seed vigour, soluble sugars, standard germination

Introduction

Sorghum and maize are important staple crops cultivated by many subsistence farmers in the semi-arid regions of the world. Among the cereal crops, sorghum and maize are considered as the principal source of energy, protein, vitamins and minerals for millions of the poor people living in these regions (Buerkert et al., 2001; Duodu et al., 2003). In many semi-arid areas these

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crops are produced almost entirely under rainfed conditions, so they invariably suffer from drought stress of varying intensity and duration in one or more growth stages. In areas where supplemental irrigation is rarely practised to avoid drought damage at critical stages of crop growth, resource-poor farmers depend largely on plants which survive drought stresses in the previous season for their food consumption and planting material. This situation is a severe threat to rural household food security, leading to chronic under-nourishment of the people. The food grains grown under such conditions form a poor quality diet, because of deficiencies of various photosynthetic assimilates.

Studies in maize have demonstrated that water deficit inhibits photosynthesis, and the decrease in photosynthate flux to the developing organs appears to trigger abortion, resulting in a decrease in grain number (Boyer and Westgate, 2004). Previously, Nicolas et al. (1985) showed that the sink size of wheat grains was reduced by drought because of the reduced number of endosperm cells and starch granules. Stem reserve mobilization has been reported to be affected by water deficits. Under dryland field conditions, only half the amount of water-soluble carbohydrates was available for remobilization during grain filling in spring wheat genotypes, as compared with irrigated conditions (Winzeler et al., 1989). Even the rate at which water deficit develops may affect mobilization (Blum, 1996). Palta et al. (1994) found that total grain carbon was reduced by 24% in plants exposed to rapidly developing water deficit compared to those where stress developed slowly.

The most visible symptoms of dehydration injury are the decrease in seed germination (Schmidhalter and Oertli, 1991) and the inhibition of growth, which is reflected in a reduction in dry matter yield (El-Tayeb and Hassanein, 2000; Le Thiec and Manninen, 2003). Under field conditions, sorghum and maize grown on subsistence farms often show poor seed germination and stand establishment, mortality and retarded growth early in the seedling stage. Vigour and germination ability are important characteristics of seed quality, since they are prerequisites for successful stand establishment and increased grain production, while the quality of grain production ensures food security for rural communities. It is thus of vital importance to know what effects, if any, water deficit during the pre- and post-flowering stages may have on seed viability and storage reserves, and how dehydration and rehydration cycles at critical growth stages influence subsequent seed performance and production. This knowledge could lead to the development of cultivars more able to efficiently partition assimilates to the reproductive organs in the field.

Materials and methods

Plant material, growth conditions and seed harvesting

Seeds of maize (*Zea mays* L.) (cv Melkassa-2) and sorghum (*Sorghum bicolor* Moench L.) (cv Macia) were obtained from the maize improvement programme for moisture stress areas, Melkassa Agricultural Research Centre, Ethiopia, and the ICRISAT Centre, Bulawayo, Zimbabwe,

respectively. The experiment was conducted in a controlled chamber under constant environmental conditions (12/12 h day/night, 28–32/17°C day/night temperature, 60–80% RH and 1200–1400 μmol $m^{-2}s^{-1}$ PPFD) at the Department of Botany, University of Cape Town. To ensure emergence, five seeds were sown in each plastic pot, each 31 cm deep with an internal diameter of 18 cm, containing about 10 kg of sandy loam soil. Emergence occurred 5–7 days after planting. Twenty days after emergence, the pots were thinned to two seedlings of uniform size per pot. The plants were watered frequently to avoid the development of moisture deficit. At 60 (pre-flowering) and 90 (post-flowering, grain-filling stage) days after emergence, two watering treatments were applied: fully hydrated (control) and dehydrated. The control plants were regularly watered to field capacity to avoid the development of water stress, while dehydration was induced by withholding water for 20 days at each growth stage. At the end of each dehydration treatment, the plants were rehydrated by soil watering (as for the control plants) for another 20 days and their recovery was studied. Three pots of each species in each treatment represented three replications. Five different samples were taken during the dehydration period at each different growth stage and during recovery. Each pot was given P and N at a rate of 0.80 g/pot (150 kg/ha) and 1.1 g/pot (200 kg/ha), respectively. Single superphosphate and lime ammonium nitrate were used as the sources of P and N. At physiological maturity (development of the black layer) (Tuinstra et al., 1997), seeds of maize and sorghum were harvested from each treatment and air-dry weight was recorded. Seeds for the standard germination and vigour tests were sun-dried (25–27°C) to a moisture content of 12%, and those for sugar, lipid and total protein content analysis were freeze-dried and stored until analysis, when triplicate extractions were performed from seeds of three separate plants.

Standard germination and vigour tests

For the germination test, four replicates of 25 seeds of both maize and sorghum were placed between a double layer of paper towels moistened with distilled water, gently rolled into a tube and covered with a plastic bag. The rolled tubes were kept upright in a dark germination chamber with a constant temperature of 25°C for 7 days. To keep the seeds moistened, the paper towel tubes were opened and watered after three to four days. Germination counts were made after 7 days. Seeds were considered germinated when the normal protrusion of the coleoptiles and radicle was visible.

For the seed vigour test, the seeds were subjected to accelerated aging by exposing them to adverse conditions of high temperature (43°C) and high relative humidity. To create high relative humidity conditions, 40 ml distilled water was placed in a plastic container, on top of which four replicates of 25 seeds of maize and sorghum from each treatment were evenly distributed on a wire mesh. The containers were then closed with a plastic lid and the accelerated ageing boxes were placed in a germination chamber adjusted to 43°C in the dark for 72 h. After accelerated ageing, the seeds were allowed to germinate in a rolled moistened paper towel following the same procedures as that of the standard germination test. Both the standard germination and seed vigour test counts were expressed as percentage germination.

Determination of soluble and insoluble carbohydrates

Freeze-dried maize and sorghum seeds (0.25–0.50 g) were ground to a fine powder in liquid nitrogen (N_2) using a pestle and mortar. Soluble sugars (sucrose, glucose and fructose) were extracted with 100 mM NaOH in 50% (v/v) ethanol. The reaction mixture was vigorously mixed, during which the tissue extracts were adjusted to pH 7–8 by adding 500 μl of 100 mM HEPES in 100 mM glacial acetic acid. The reaction mixture was centrifuged at 16,000 rpm in a Beckman J2-21 centrifuge for 20 min at 4°C. The pellets were re-extracted using the same procedure as described above and the two supernatants from each extraction were combined.

Quantification of the soluble sugars was done enzymatically using a Boehringer Mannheim sugar food analysis kit (Bergmeyer and Bernt, 1974). The amount of NADPH from the reaction of glucose-6-phosphate with NADP + glucose-6-dehydrogenase was measured spectrophotometrically at 340 nm. Fructose was converted to glucose-6-phosphate in two steps using hexokinase and glucose phosphate isomerase, while sucrose was measured as the additional amount of D-glucose formed after inversion with β-fructosidase (D-glucose being measured as described above).

The starch concentration was colorimetrically determined using the method described by Buysse and Merckx (1993). Maize and sorghum seed samples (0.25 g) were ground to a fine powder in liquid N_2 using a pestle and mortar. After drying the sample used in sugar extractions, the extraction of starch was carried out with 10 ml of 32% HCl. The sample extract was incubated in a boiling water bath for 3 h and centrifuged at 25,000 g in a Beckman J2–21 centrifuge for 5 min at 4°C. The starch concentration was determined using a reaction mixture containing 1 ml phenol, 5 ml H_2SO_4 and 0.5 ml sample. A blank (without added sample) was measured at the beginning. A standard curve with glucose concentrations in the range of 20–80 µg/ml was established.

Determination of lipid

Approximately 1 g of maize and sorghum seeds was finely ground with a mortar and pestle using liquid N_2. A volume of 25–40 ml of chloroform : methanol (v/v 2:1) was added and homogenized with Ultra-Turox homogenizer. The sample extract was centrifuged and the clear supernatant was carefully removed using an automatic pipette into a measuring cylinder and the total volume made up to 60 ml with the extraction medium. To remove polar contaminants, the extracts were transferred to a separating funnel and a Folch wash was carried out, comprising of 15 ml 0.88% KCl solution. After shaking the contents of the separating funnel together, the two phases were allowed to separate. The lower organic phase containing lipids was then removed by running it into a pre-weighed glass vessel, after which it was concentrated under a vacuum in a Savant SpeedVac SC110 rotary concentrator. The samples were stored in a desiccator and weighed daily until constant mass was attained. The total lipid content was expressed as mg lipid/g dry weight.

Determination of total seed protein

Approximately 0.1 g of freeze-dried maize and sorghum seeds was weighed and ground to a fine powder with a pestle and mortar in liquid N_2. A volume of 3 ml extraction buffer containing 30 mM TES (pH 7.5), 20 mM NaCl and 1 mM PMSF was used for the extraction of total protein. The sample extract was shaken for 15 min and centrifuged at 10,000 rpm for 15 min at 4°C. A 40 µl volume of protein supernatant was then mixed with 2 ml of diluted Coomassie Blue G-250 reagent, prepared according to the method of Bradford (1976) in 96% ethanol and 85% phosphoric acid. Absorbance was read at 595 nm in a Beckman DU650 (USA) spectrophotometer. The concentration of protein in the sample was calculated from a standard curve prepared from known masses of bovine serum albumin (BSA).

Statistical analysis

Statistical analyses were carried out using STATISTICA for Windows Version 6.0 (Statsoft Inc., USA). The results presented were the means of three replicates. In all figures, means were calculated and significant differences between treatments and between the two species were tested by factorial analysis of variance and Duncan's multiple range test at the 5% level of significance. Standard errors are represented as vertical bars.

Results

Standard germination and vigour

The decrease in percentage germination of seeds harvested from pre- and post-flowering dehydrated plants varied with the species. The percentage germination of maize seeds from plants dehydrated during the two developmental stages decreased significantly ($P<0.05$) to 17% and 28% after pre- and post-flowering treatment as compared to the control (Fig. 1a), while that of sorghum seeds harvested from plants dehydrated during the pre- and post-flowering stages decreased to only 82% and 75% relative to the control.

The germination of control seeds of maize and sorghum was not affected by accelerated ageing relative to the standard germination control seeds (Fig. 1b), indicating their tolerance to adverse environmental conditions. The germination of maize seeds was poor after dehydration, irrespective of accelerated aging. In the case of sorghum, however, accelerated aging had no significant ($P<0.05$) effect on the vigour of seeds harvested from pre-flowering dehydrated plants, while the percentage germination declined significantly when accelerated aging was followed by post-flowering dehydration (Fig. 1b).

Soluble and insoluble carbohydrates

The contents of soluble and insoluble sugars in the seeds of the two species during pre- and post-flowering dehydration are given in Figure 1c and d. The data showed that the sucrose and glucose contents of maize seeds decreased significantly ($P<0.05$) and in a similar manner in response to both pre- and post-flowering dehydration. Dehydration during the post-flowering stage induced a significant ($P<0.05$) increase (47%) in the sucrose content as opposed to a significant decrease in the case of pre-flowering dehydration.

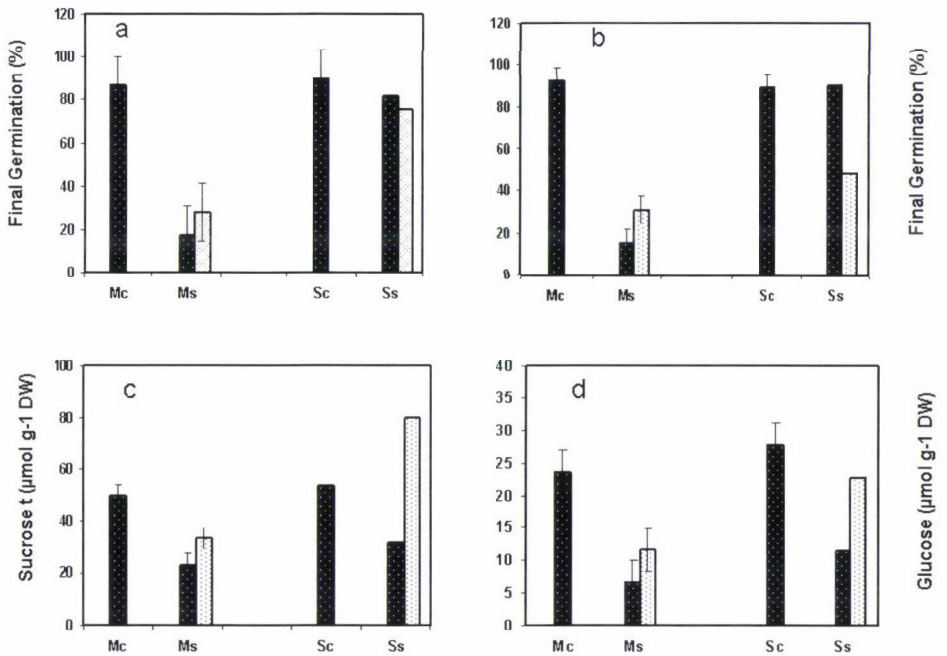

Fig. 1. Germination (a), germination after accelerated ageing (b), sucrose content (µmol g^{-1} DW) (c) and glucose content (µmol g^{-1} DW) (d) of maize and sorghum seeds harvested from control plants and from plants dehydrated during the pre- and post-flowering stages. Vertical bars denote the standard error ($P<0.05$) of means (n=4). Black and white columns represent the pre- and post-flowering stages, respectively. Mc and Sc denote control treatments and Ms and Ss dehydrated treatments for maize and sorghum, respectively

The glucose content showed no significant change in sorghum seeds subjected to pre-flowering dehydration compared to the control seeds, whereas it decreased significantly in those subjected to post-flowering dehydration. In maize seeds the sucrose content decreased by 47% and 33% during pre- and post-flowering dehydration, respectively, while the decrease in the glucose content (52% and 61%, respectively, in the case of pre- and post-flowering dehydration) was significantly higher than that recorded for sorghum, which exhibited a decrease of 27% and 21% in response to pre- and post-flowering dehydration, respectively.

In maize seeds, the fructose content decreased significantly ($P<0.05$) by 70% and 51% in response to pre- and post-flowering dehydration, respectively (Fig. 2a), while in sorghum the decline was only significant for seeds subjected to pre-flowering dehydration (56%), being only 19% after post-flowering dehydration.

The starch contents of the two species declined significantly ($P<0.05$) and followed similar trends when subjected to pre- and post-flowering dehydration (Fig. 2b).

Lipid

In general, the lipid content of maize seeds from control plants was higher than that of sorghum seeds (Fig. 2c). Dehydration significantly ($P<0.05$) decreased the lipid contents of both maize and sorghum seeds harvested from pre- and post-flowering dehydrated plants relative to the control seeds, but there was no significant ($P<0.05$) difference in lipid contents between the species. In both species pre-flowering dehydration caused a significantly ($P<0.05$) greater decrease than post-flowering dehydration.

Seed protein

There were considerable differences between the species in the total protein content of seeds harvested from control and dehydrated plants (Fig. 2d), with much higher values for sorghum in all cases. In general, dehydration had a significant ($P<0.05$) effect on the protein content of both species, but this effect differed for the species and for the stage at which dehydration treatment was applied. In maize, the protein content of seeds harvested from pre-flowering dehydrated plants markedly decreased, while that of seeds from post-flowering dehydrated plants increased relative to the control. In contrast, there was an increase in protein content in sorghum seeds harvested from pre-flowering dehydrated plants, and a decrease in seeds from post-flowering dehydrated plants.

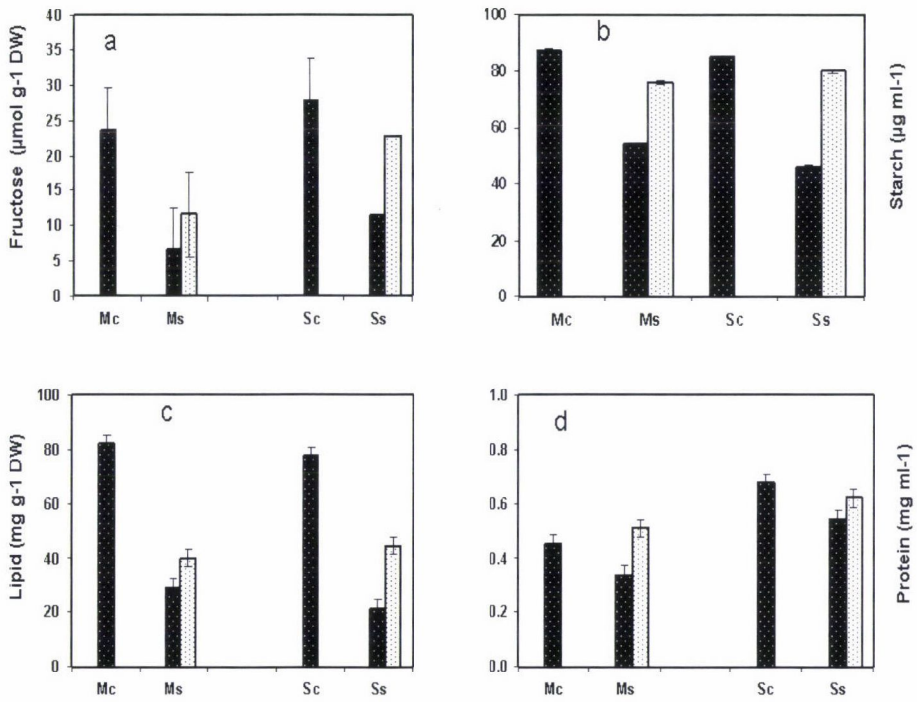

Fig. 2. Fructose content (µmol g^{-1} DW) (a), starch content (µmol g^{-1} DW) (b), lipid content (mg g^{-1} DW) (c) and protein content (mg ml^{-1}) (d) of maize and sorghum seeds harvested from control plants and plants dehydrated during the pre- and post-flowering stages. Vertical bars denote the standard error ($P<0.05$) of means (n=4). Black and white columns represent the pre- and post-flowering stages, respectively. Mc and Sc denote control treatments and Ms and Ss dehydrated treatments for maize and sorghum, respectively

Discussion

Dehydration is known to trigger a wide variety of plant responses, ranging from physiological, cellular and biochemical responses to changes in growth rates and crop yields. In this study, the indices investigated exhibited variable responses to dehydration in both species (Fig. 1).

There was variation in the response of maize and sorghum to dehydration in the percentage germination and vigour after accelerated ageing (Fig. 1), the greatest reduction being observed in maize seeds, irrespective of whether dehydration was suffered during the pre- or post-flowering stage. This suggests that the development of maize grains is more susceptible to both pre- and post-flowering dehydration than that of sorghum seeds. According to Roberts (1972), the decrease in essential metabolites, including the loss of precursors for storage reserves (such as free sugars), is an important factor in the loss of seed viability and vigour. Similar results have been reported by other authors on different

plants (El-Tayeb and Hassanein, 2000; Schütz et al., 2002). Sorghum seeds, however, showed an age-dependent difference in vigour after accelerated ageing (Fig. 1). The reduction in the vigour of sorghum seeds harvested from post-flowering dehydrated plants after exposure to high temperature and high % RH suggests that the effect of poor storage conditions could lead to substantial reductions in vigour and seedling establishment under field conditions in plants subjected to drought during the post-flowering stage. These findings are in accordance with the results obtained for other seed species (Lin and Pearce, 1990; Ray et al., 1990; Basavarajappa et al., 1991; Gurmu and Naylor, 1991; Madhava Rao and Kalpana, 1994).

It is well known that dehydration influences the carbohydrate concentration of crop plants. In both species, dehydration during the pre- and post-flowering stages resulted in significant reductions in soluble and insoluble sugar deposition, but the effect was more marked when dehydration occurred during the pre-flowering stage (Figs. 1 and 2). This is in agreement with the findings of Westgate and Thomson Grant (1989), who reported that the level of carbohydrate concentration in maize was more severely affected by water deficit during anthesis than in the mid-grain-filling stage. Dehydration during the pre-flowering stage coincides with zygote formation, endosperm cell division and early grain growth, which are clearly sensitive to severe dehydration, and developmental failure at low relative water contents has often been observed (Schoper et al., 1987; Westgate and Thomson Grant, 1989). Subsequently, the sink size of the grains might have been reduced by dehydration, because of the lower number of endosperm cells. It is possible, therefore, that the decrease in soluble sugars in pre-flowering dehydrated sorghum seeds was a consequence of the low demand for assimilates by the main sink (developing grain).

The ultimate carbon source for the formation of storage lipids by developing seeds is usually sucrose (Murray, 1984). The decrease observed in storage lipids in maize seeds harvested from pre- and post-flowering dehydrated plants and in sorghum seeds harvested from pre-flowering dehydrated plants (Fig. 2) suggests that sucrose might have been unavailable in sufficient amounts for the biosynthesis of lipids, since the sucrose concentration in these seeds was low. Sorghum seeds harvested from post-flowering dehydrated plants might be expected to maintain relatively high concentrations of storage lipids due to their increased concentration of sucrose. It appears, however, that the deposition of storage lipids ceased as a result of dehydration during the final stage of grain filling, and the enzymes involved might have been destroyed and their synthesis stopped (Bewley and Black, 1994).

It has frequently been reported that the percentage of protein or nitrogen in the grain is increased by plant water deficits (Brooks et al., 1982; Barber and Jessop, 1987). In severe cases, however, the translocation of nitrogen may be inhibited to the extent that mobilized amino acids become trapped in senescing leaf tissues (Tully et al., 1979). The protein contents of seeds subjected to dehydration pre-flowering (sorghum) and post-flowering (maize and sorghum)

confirmed these findings. This result is in agreement with the findings of Brooks et al. (1982), who reported an increase in the amino acid concentration of barley seeds due to water deficit at the grain-filling stage. The protein concentration was only significantly reduced in maize seeds subjected to pre-flowering dehydration (Fig. 2), possibly due to the inhibition of nitrogen remobilization as a result of the rapid development of water deficit. Jenner et al. (1991) suggested that the rapid onset of water stress severely curtailed nitrogen remobilization through translocation effects.

Conclusions

This study demonstrated that dehydration at the pre- and post-flowering stages influenced the grain yield and seed quality (standard germination and vigour) of both maize and sorghum. Maize seeds, however, were more susceptible to dehydration, irrespective of the developmental stage, than sorghum seeds. Sorghum appeared to lose vigour only when dehydration occurred during the post-flowering stage. The ability of sorghum seeds harvested from plants grown under adverse environmental conditions to germinate could give sorghum an advantage in stand establishment under field conditions. The germination ability of seeds under both ideal and adverse conditions is very important for stand establishment in semi-arid conditions.

The capacity to remobilize reserve assimilates to the growing grains under drought stress has been recognized as a dehydration tolerance process in crop plants. The better ability of sorghum plants to remobilize sugars, starch, lipids and proteins than maize could make sorghum a desirable crop for semi-arid areas.

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A SIMPLIFIED METHOD TO TEST CEREAL FROST TOLERANCE

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Testing cereal frost tolerance goes back for decades in the Agricultural Research Institute, Martonvásár, Hungary. The climatic programmes used in the plant growth chamber have proved to be fairly efficient, but these methods are time-consuming and have become quite expensive in recent years. An attempt was made to shorten this process by reducing the cold hardening phase, and the freezing test has been simplified and shortened by measuring the relative conductance of leaf segments frozen in a liquid freezer. Frost-tolerant and sensitive wheat lines were tested, and the sensitivity of the system was checked by testing single chromosome substitution lines. Differences were found for all lines frozen at different temperatures. To reduce the costs of the experiment it was attempted to cold-harden the plants not only in a growth chamber but also in a cold room under very low light intensity and it was found that even under thess unfavourable conditions the plants developed a certain level of frost tolerance. The simplified frost tolerance test has proved to be effective, but requires further improvement due to the unsatisfactory significance levels.

Key words: frost tolerance, cold hardening, relative conductance, electrolyte leakage, wheat, cereal

Abbreviations: CS, *Triticum aestivum* ssp. *aestivum* cv. Chinese Spring; CNN, *Triticum aestivum* ssp. *aestivum* cv. Cheyenne; CS/CNN5A, Chinese Spring/Cheyenne 5A; CS/TSP 5A, Chinese Spring/*Triticum spelta* 5A; RC, relative conductance

Introduction

Frost tolerance is a complex character, being influenced not only by physiological and genetic factors, but also by environmental factors. Many methods have been developed to test cereal frost tolerance. These differ in the way plants are sown, grown, cold-hardened and subjected to freezing and in the way frost damage is estimated. Frost tests can be carried out in the field or in growth chambers. The advantage of tests in a controlled environment is obvious:

they are more reproducible. Moreover, many freezing temperatures can be applied in experiments run in growth chambers in order to differentiate the cereal genotypes on the basis of frost resistance. The evaluation of frost tests can be done by direct or indirect methods. Indirect methods, such as the measurement of tissue water content or proline accumulation, or *Cor* (cold regulated) gene expression analysis are based on the measurement of phenomena not directly related to frost damage. On the other hand, direct methods mostly estimate physiological changes that are due to, or closely related to, the effect of frost itself. A detailed description of various testing methods was given by Reynolds et al. (2001). These include the determination of plant survival, the study of photosynthetic system integrity by chlorophyll fluorescence analysis and the estimation of cell membrane damage by electrolyte leakage (conductance) measurements. The use of conductance measurements to assess freezing injury was studied in detail by Prášil and Zámečik (1998). This method has been successfully used to study the frost tolerance of various species, such as the model plant *Arabidopsis* (Miuraa et al., 2007), or cereals, for example common and durum wheat or rye (Limin and Fowler, 1988).

Winter cereals planted in autumn must survive freezing temperatures during the winter. Two mechanisms play a central role in the adaptation of temperate cereals to freezing temperatures. The first, vernalization, is the delay of flowering until the end of the winter to protect the sensitive floral primordia after long-term exposure to cold temperatures (reviewed by Trevaskis et al., 2007; Distelfeld and Dubcovsky, 2010). The second mechanism, known as cold acclimation or cold hardening, improves the resistance of the vegetative tissues to freezing damage (Thomashow, 1990; 1999). After an initial increase in freezing tolerance, winter genotypes exhibit a progressive decrease in their cold acclimation ability (Fowler et al., 1996). This decrease inversely parallels the fulfilment of the vernalization requirement and plant development (Fowler et al., 1996; Limin and Fowler, 2006).

In the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, several standard protocols are used for plant growth, cold hardening and freezing tests (Tischner et al., 1997). Sutka (1981) developed a climatic programme (FDA) which proved to be reliable in many experiments. Based on this programme, a "classical" frost test is symbolized in Figure 1. Seeds are sown in wooden boxes, which are placed in growth chambers. The germination and growth period lasts for some 20 days with gradually decreasing temperatures. The subsequent cold hardening period lasts for 3 weeks, while freezing is a five-day process carried out in freezing chambers. After freezing the plants are cut back several cm above the soil, and at the end of a two-week re-growth period the frost tolerance is evaluated either as the percentage of survival or by assessing the re-growth of the plants, scored on a 0 (death) to 5 (undamaged) scale. The whole freezing process lasts for ten weeks, so it is rather time-consuming. Considering the rise in energy costs in recent years it has also

become quite expensive. These considerations raised the need for a simplified, faster and cheaper method to test cereal frost tolerance. Attempts were made to modify the growth and cold hardening periods. The freezing processes were carried out in a liquid freezer, making it possible to apply several frost testing temperatures in a single experiment, and the whole freezing period was no longer than one day. Moreover, the method involves measuring the electrolyte leakage from plant cell tissues caused by frost damage, so there is no need for a two-week recovery phase, as in "classical" testing methods, again reducing the costs of the experiment.

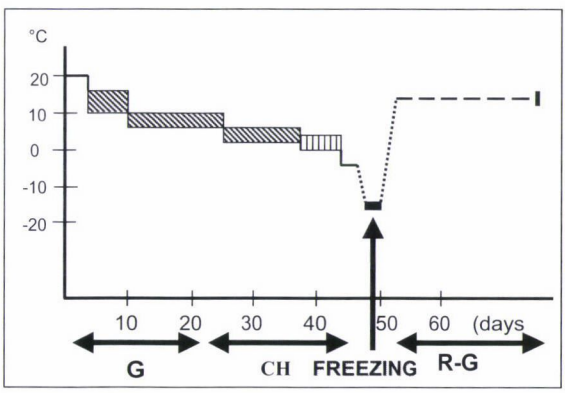

Fig. 1. Schematic representation of a "classical" frost test.
(G: Growth, CH: Cold hardening, R-G: Re-growth)

Materials and methods

Plant material

Triticum aestivum lines with a known level of frost tolerance were used. The frost-sensitive line Mv 8, the spring variety *Triticum aestivum* ssp. *aestivum* cv. Chinese Spring (frost-sensitive), and the highly frost-tolerant *Triticum aestivum* ssp. *aestivum* cv. Cheyenne were studied. To test the sensitivity of the system, two single chromosome substitution lines, Chinese Spring/Cheyenne 5A (frost-tolerant) and Chinese Spring/*Triticum spelta* 5A (frost-sensitive) were also included.

Plant growth and cold hardening

The seeds were sown in wooden boxes and transferred to growth chambers (Conviron, Canada). In the first experiment the growth period lasted for 16 days at 17/13°C (day/night) with 260 $\mu m/m^2s$ illumination, after which the temperature was gradually decreased (13/10° and 8/6°C, each for one day). Then the plant material was divided to test the effect of different cold hardening circumstances. The first set was cold-hardened in a growth chamber for 18 days, while the second set was transferred to a vernalization room for 18 days. The temperature was +4°C in both cases, but the light intensity in the vernalization room was only 1/10 (30 $\mu m/m^2s$) of the intensity applied in the growth chamber (260 $\mu m/m^2s$).

In the second experiment (growth: 17/13°C, 260 $\mu m/m^2s$, 16 days) the plants were cold-hardened for 35 days in the vernalization room (+4°C, 260 $\mu m/m^2s$). In the third experiment the plants were grown for 35 days (20/20°C, 260 $\mu m/m^2s$) and cold hardening was carried out in the growth chamber at 4/4°C for 20 days.

Freezing

After cold hardening, leaf segments (all the same size, approximately 3 cm long) were collected. In order to avoid mechanical damage during wrapping, the leaf segments were placed on slightly larger plastic sheets, wrapped in aluminium foil, and then inserted into glass test tubes. To ensure sufficient thermal conduction the tubes were filled with dry sand. Freezing was carried out in a computer-controlled liquid freezer (GP200-R4 circulating bath, Grant Instruments Ltd., Cambridge, UK) previously set to +2°C. The leaf segments were kept at +2°C for 2 h, then subjected to a second phase of cold hardening at –2°C for 14 hours. The temperature was then decreased by 2°C/h to the freezing temperature (–4, –6, –8, –10, –12, –14, –16°C). Each freezing temperature was maintained for one hour in the first and second experiments and for two hours in the third, and then quickly reduced to the next lower value. To collect frozen samples at each freezing temperature, one set of samples was removed and allowed to thaw at 2°C for two hours, while the rest of the samples were left in the freezer for further freezing at lower temperatures.

Conductance measurements

Thawed leaf segments were carefully unwrapped and placed in Falcon tubes filled with 10 ml Milli-Q water. The tubes were continuously shaken (300 RPM) for 24 hours at room temperature. Six unfrozen leaf segments were boiled for 45 minutes to estimate the maximum electrolyte leakage, and the conductance of three unfrozen leaf segments was also measured for each genotype tested. Conductance was measured using a MultiSample Conductometer (Mikro KKt., Hungary) on an average of 10 plants per genotype × temperature combination. Relative conductance was calculated using the equation:

Relative conductance = (Conductance of frozen sample – Average conductance of unfrozen samples) / Average maximum conductance × 100.

Results

In the first experiment the plants were grown for 16 days, then cold hardened at different illumination levels at 4/4°C (day/night) for 18 days. Half the plants were illuminated at the frequently applied level of 260 $\mu m/m^2s$ in a growth chamber, while the rest of the material was placed in a vernalization room lit only at 30 $\mu m/m^2s$. The vernalization room was used since this equipment is operated continuously at a standard temperature, at only a fraction of the cost of a growth chamber. After cold hardening, leaf segments were collected and frozen in a liquid freezer at –8, –10, –12, –14 and –16°C for one hour at each temperature. Then electrolyte leakage was measured and the frost tolerance was evaluated as a percentage of maximum conductance.

In the case of plants cold-hardened in a growth chamber, differences were found between the three wheat genotypes at all the test temperatures. The lowest level of relative conductance (least electrolyte leakage) was recorded for the most frost-tolerant genotype Cheyenne at all the temperatures, while the highest values were detected for the least tolerant variety Chinese Spring (only exception: –14°C). As expected, lower values were detected at higher temperatures (Fig. 2A).

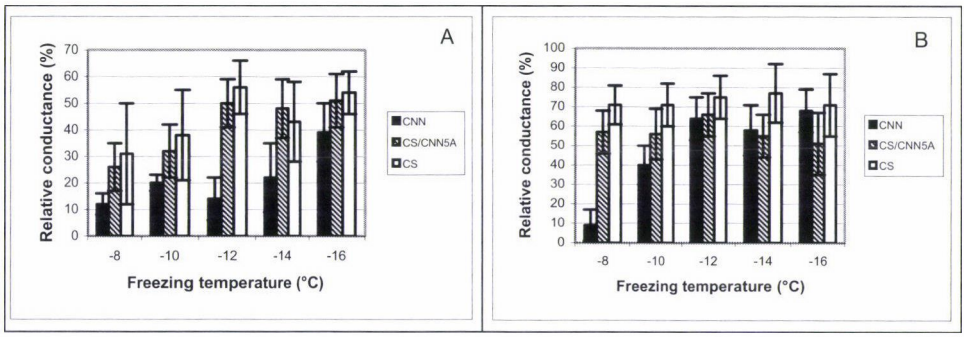

Fig. 2. Relative conductance (ion leakage) of leaf segments tested at different freezing temperatures. (A: Plants were cold-hardened for 18 days in a growth chamber under a light intensity of 260 μm/m^{2}s; B: Plants were cold-hardened for 18 days in a vernalization room under a light intensity of 30 μm/m^{2}s)

Genotype-dependent relationships were detected at higher freezing temperatures (–8, –10 and –12°C) for plants cold-hardened at low light intensity. However, this correlation was not detected when the leaves were frozen at low (–14, –16°C) temperatures (Fig. 2B).

To study the effect of the duration of low light intensity a frost-tolerant (Cheyenne) and a frost-sensitive variety (Mv 8) were cold-hardened under low light (30 μm/m^{2}s) intensity for 35 days in the vernalization room. It was found that at higher freezing temperatures (–6, –8°C) the relative conductance (RC) values were close to zero for both varieties. However, as the freezing temperature decreased (–10, –12, –14°C) these values gradually increased, and clear differences were found between the two varieties (Fig. 3).

To check the sensitivity of the system, besides Cheyenne (CNN) and Chinese Spring (CS) two single chromosome substitution lines, the frost-tolerant Chinese Spring/Cheyenne 5A (CS/CNN5A) and the frost-sensitive Chinese Spring/*Triticum spelta* 5A (CS/TSP5A) were also studied. At –6°C none of the genotypes were injured. At –8°C the most sensitive line (CS/TSP5A) showed far the highest RC value. At –10°C the frost-sensitive CS exhibited a great increase in RC (Fig. 4A). The frost-resistant lines (CNN and CS/CNN5A) showed a much slighter increase compared to the resistant genotypes at all the testing temperatures (Fig. 4B).

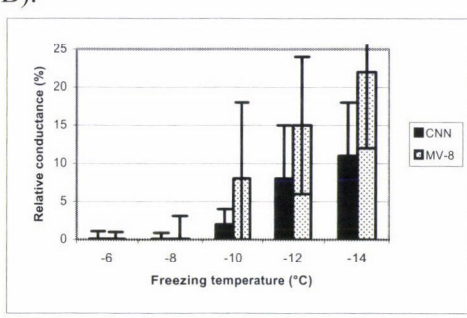

Fig. 3. Relative conductance of leaf segments tested at different freezing temperatures. Plants were cold hardened for 35 days under low illumination

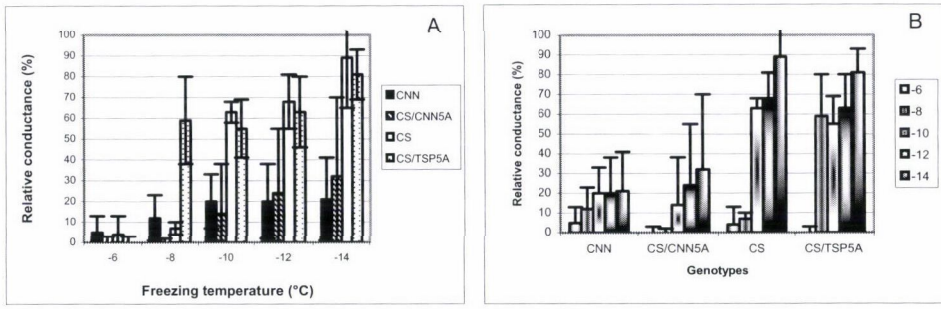

Fig. 4. Relative conductance of the four genotypes tested. A: Relative conductance in relation to freezing temperatures, B: Effect of decreasing the freezing temperature on the genotypes

Discussion

In a study on the kinetics of the cold hardening process, Vágújfalvi et al. (1999) compared the level of frost tolerance of frost-sensitive and resistant wheat lines and found that the frost-resistant Cheyenne variety started to develop frost tolerance in the early phase of hardening, reaching the maximum level after 11 days, while the frost-sensitive Chinese Spring reach a maximum only on the 43rd day. The substitution of chromosome 5A from Cheyenne to Chinese Spring increased the frost tolerance of the recipient line and the maximum tolerance was reached earlier, on the 30th day of cold acclimation. This experiment also highlighted the clear differences that can be observed between the genotypes in the earlier phase of hardening, i.e. the induction of resistance was delayed in the sensitive lines. In the present experiment the cold hardening period was reduced. In spite of the fact that the plants were not fully cold-hardened, it was possible to demonstrate genotype-dependent frost tolerance.

During the cold hardening period, light is an essential environmental factor to develop a sufficient level of frost resistance (Szalai et al., 2009). Under normal illumination (260 μm/m^{2}s) the frost tolerance was found to decrease (relative conductance increased) as the freezing temperature dropped (Fig. 2A). However, when the plants were cold-hardened for 18 days under low light intensity (Fig. 2B) genotype-dependent frost tolerance was only detected at higher (–8, –10 and –12°C) temperatures. Under more severe freezing conditions (–14, –16°C) there were no meaningful differences. It is suggested that this low light level was unable to induce sufficient tolerance to withstand the lowest temperatures even in more resistant lines. This hypothesis is supported by the fact that when the plants were cold hardened under the same low illumination for 35 days, the level of frost tolerance was found to be genotype-dependent (Fig. 3) even at lower temperatures.

This simplified frost tolerance test has proved to be effective, but requires improvement due to the insufficient significance levels. This could be achieved either by increasing the sample number or by more uniform sampling size. The method was sensitive enough to discriminate frost-tolerant and sensitive wheat

genotypes, and to demonstrate the effect of a single chromosome on the level of frost tolerance. The freezing parameters and the sampling procedure were adjusted so that they could be completed in an eight-hour working day, and the whole freezing process and conductance measurement can be completed in less than 3 days.

Acknowledgements

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PRODUCTION AND FISH IDENTIFICATION OF WHEAT–*Aegilops biuncialis* ADDITION LINES AND THEIR USE FOR THE SELECTION OF U AND M GENOME-SPECIFIC MOLECULAR (SSR) MARKERS

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One way of incorporating useful traits from *Aegilops biuncialis* (2n=4x=28, $U^bU^bM^bM^b$) into wheat (*Triticum aestivum* L. 2n=6x=42, AABBDD) is to develop first addition then translocation lines. The $2M^b$, $3M^b$, $7M^b$, $3U^b$, $5U^b$ and $5U^b/6U^b$ wheat–*Ae. biuncialis* addition lines were produced in Martonvásár. To facilitate the exact identification of the addition lines, it was necessary to analyse the fluorescence *in situ* hybridisation patterns of the parental wheat genotype, *Ae. biuncialis* and its diploid progenitors (*Ae. umbellulata* 2n=2x=14, UU and *Ae. comosa* 2n=2x=14, MM). The great genetic variability of the *Aegilops* species causes polymorphism in the fluorescence *in situ* hybridisation (FISH) patterns of the individual chromosomes. Due to the high level of FISH polymorphism, it is advisable to confirm the identification of the *Ae. biuncialis* chromosomes with the help of molecular (microsatellite, SSR) markers, so 119 wheat SSR markers were tested on *Aegilops biuncialis*, on *Ae. geniculata* (2n=4x=28, $U^gU^gM^gM^g$), on five wheat–*Ae. biuncialis* addition lines ($2M^b$, $3M^b$, $7M^b$, $3U^b$, $5U^b$) and on an addition series of wheat–*Ae. geniculata* in order to select SSR markers specific to the U and M genomes of *Ae. biuncialis* and *Ae. geniculata.*

Key words: *Ae. biuncialis*, *Ae. geniculata*, addition lines, *in situ* hybridisation, SSR markers

Introduction

Aegilops biuncialis Vis. (2n=4x=28, $U^bU^bM^bM^b$), a wild allotetraploid species closely related to cultivated wheat, carries numerous resistance genes (for review see Schneider et al., 2008). Some accessions of *Ae. biuncialis* are tolerant of salt (Colmer et al., 2006) and drought (Molnár et al., 2004). Their ability to spread widely was possibly due to their high adaptability. Several wheat–*Aegilops* addition and translocation lines have been developed and many agronomically useful traits have been incorporated into the wheat genome, but there have been no reports yet on gene transfer from *Ae. biuncialis* into wheat. Agronomically useful genes from *Ae. biuncialis* can be transferred into wheat via intergeneric crossing following the production of addition and translocation

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lines. The aim of the experiments was to identify the wheat–*Ae. biuncialis* addition lines produced in Martonvásár. *In situ* hybridisation (ISH) is a powerful tool for the detection and identification of alien chromosomes or chromosome segments. In order to identify the alien (*Ae. biuncialis*) chromosomes in different genetic materials it is necessary to use fluorescence *in situ* hybridisation (FISH), while possible chromosome rearrangements between different genomes can be detected using genomic *in situ* hybridisation (GISH). To facilitate the FISH identification of wheat–*Ae. biuncialis* addition lines, it was necessary to analyse the FISH patterns of parental wheat and *Ae. biuncialis* accessions.

Aegilops species have great genetic diversity, which causes substantial polymorphism in the FISH patterns of the individual chromosomes (Schneider et al., 2005). This complicates the detection of the *Aegilops biuncialis* chromosomes in the wheat genome, making the alien chromosomes difficult to identify. On the grounds of the high level of FISH polymorphism, it is useful to support the identification of the *Ae. biuncialis* chromosomes with the help of molecular (microsatellite, SSR) markers. Up till now very few chromosome-specific microsatellite markers have been described in *Aegilops* species (Lelley et al., 2000; Dhaliwal et al., 2002; Zaharieva et al., 2003; Adonina et al., 2005), but the number of SSR markers available for the U and M genomes is particularly limited (Dhaliwal et al., 2002; Zaharieva et al., 2003). Numerous chromosome-specific SSR markers have been reported for cultivated wheat (Röder et al., 1998; Pestsova et al., 2000; Somers et al., 2004; Song et al., 2005), some of which also give bands on *Ae. biuncialis*, due to the close relationship between wheat and *Ae. biuncialis*. The aim of the experiment was to select U and M genome-specific SSR markers, making it possible to detect *Ae. biuncialis* chromosomes in the genetic materials produced in Martonvásár. The first task was to select markers that gave a length polymorphic band on *Ae. biuncialis* compared with wheat, after which these markers were selected for chromosome specificity on wheat–*Ae. biuncialis* (Molnár-Láng et al., 2002; Schneider et al., 2005) and wheat–*Ae. geniculata* (Friebe et al., 1999) addition lines.

Materials and methods

The plant material consisted of four accessions each of *Ae. umbellulata* (TA1829, TA1831, TA1835; WGRC, Manhattan, Kansas; and MvGB420; Martonvásár Gene Bank) and *Ae. comosa* (TA2101, TA1965, TA1968 and TA1975; WGRC, Manhattan, Kansas), three accessions of *Ae. biuncialis* (MvGB642, MvGB382 and MvGB376; Martonvásár), the wheat genotypes Chinese Spring and Mv9 kr1 (Molnár-Láng et al., 1996), 22 wheat cultivars of various origins (for details see Schneider et al., 2003), *Triticum aestivum* cv. Mv9 kr1–*Ae. biuncialis* (MvGB642) addition lines produced in Martonvásár (Logojan and Molnár-Láng, 2000; Molnár-Láng et al., 2002; Schneider et al., 2005) and a *Triticum aestivum* cv. Chinese Spring–*Ae. geniculata* (TA2899) addition series (Friebe et al., 1999).

FISH was carried out according to Szakács and Molnár-Láng (2007) and GISH following the instructions of Molnár et al. (2009).

The repetitive DNA probes used for FISH were: pSc119.2 (Bedbrook et al., 1980), pAs1 (Rayburn and Gill, 1986).

Total genomic DNA of *Ae. umbellulata* (2n=2x=14, UU) or *Ae. comosa* (2n=2x=14, MM) was labelled with biotin or digoxigenin and detected with streptavidin-FITC (green) or antidig-rhodamin (red) (Molnár et al., 2009).

A total of 119 wheat SSR markers were analysed. The PCR reaction was carried out in an Eppendorf Mastercycler (Eppendorf-Netheler-Hinc Inc.) according to Nagy et al. (2003) with minor modifications. Agarose gel electrophoresis was carried out using 2% agarose gels.

Results

One accession of *Ae. biuncialis,* MvGB642, was crossed as a male parent with the winter wheat Martonvásári 9 kr1 (Mv9 kr1), which contains a recessive crossability gene 'kr1' incorporated from the wheat Chinese Spring (Molnár-Láng et al., 1996). F_1 hybrids with 35 chromosomes ($ABDU^bM^b$, 2n=5x=35) were produced and treated with colchicine to develop amphiploid plants ($AABBDDU^bU^bM^bM^b$, 2n=10x=70), which were backcrossed two or three times with wheat (Mv9 kr1) following one or two generations of selfing. Monosomic additions (AABBDD + $1M^b$ or $1U^b$) were selected and self-pollinated. Plants with 44 chromosomes were selected and analysed using *in situ* hybridisation.

In order to identify the alien (*Ae. biuncialis*) chromosomes in the addition lines it was necessary to analyse the FISH patterns of the parental Mv9 kr1 wheat and *Ae. biuncialis* accession, to compare them with the diploid progenitors (*Ae. umbellulata* and *Ae. comosa*). Twenty-two wheat cultivars of different origin were analysed using FISH with the help of the repetitive DNA probes pSc119.2 and pAs1. The FISH pattern of Chinese Spring was compared to that of the other wheat accessions analysed. Differences were observed in the hybridisation patterns of chromosomes 4A, 5A, 1B, 2B, 3B, 6B, 7B, 1D, 2D, 3D and 4D. The FISH technique was applied using the pSc119.2 and pAs1 DNA probes on four accessions of *Ae. umbellulata*, four accessions of *Ae. comosa* and three accessions of *Ae. biuncialis*. Most of the *Ae. umbellulata, Ae. comosa* and *Ae. biuncialis* chromosomes showed hybridisation patterns similar to the standard karyotypes (Badaeva et al., 1996), but FISH polymorphism was observed in all the chromosomes. The hybridisation patterns of *Ae. comosa* were more variable than those of *Ae. umbellulata*. The FISH patterns of *Ae. biuncialis* accessions were more polymorphic, and differences were detected not only between the *Ae. biuncialis* accessions, but also between *Ae. biuncialis* and its diploid progenitors.

Seven different disomic addition lines were produced and analysed using *in situ* hybridisation. FISH was carried out on root tip squash preparations using the repetitive DNA probes pSc119.2 and pAs1. With the help of these repetitive DNA probes the $2M^b$, $3M^b$, $7M^b$, $3U^b$, $5U^b$ and double $5U^b/6U^b$ disomic addition lines were identified (Fig. 1). In the first wheat–*Ae. biuncialis* addition line, the arm ratio and FISH pattern of the *Ae. biuncialis* chromosome pair corresponded to those of the $2M^b$ chromosome (Fig. 1). The second wheat–*Ae. biuncialis* addition line carried an extra *Ae. biuncialis* 3M chromosome pair. The FISH

pattern of chromosome 3M resembles that of the $2M^b$ chromosome, but the arm ratio is different (Fig. 1). The $7M^b$ *Ae. biuncialis* chromosome in the third wheat–*Ae. biuncialis* addition line is one of the few nearly metacentric chromosomes of *Ae. biuncialis*, with a distinctive hybridisation pattern, as can be seen in Fig. 1. In the fourth wheat–*Ae. biuncialis* addition line, chromosome $3U^b$ was identified on the basis of the FISH pattern and the arm ratio. This chromosome is nearly acrocentric, with pSc119.2 sites on both ends (Fig. 1). The fifth wheat–*Ae. biuncialis* addition line carried chromosome $5U^b$, which is a relatively small satellited chromosome with pSc119.2 sites on both ends (Fig. 1). The sixth wheat–*Ae. biuncialis* addition line contained a pair of $5U^b$ chromosomes and an acrocentric $6U^b$ chromosome pair (Fig. 1). Line No. 49.001102 carries a submetacentric *Ae. biuncialis* chromosome pair. After FISH using the pSc119.2 and pAs1 repetitive DNA probes no hybridisation site was observed on the *Ae. biuncialis* chromosome pair. GISH showed that this chromosome pair belonged to the M^b genome. No chromosome rearrangements were detected between wheat and *Ae. biuncialis* chromosomes in any of the addition lines using GISH. The spike morphology of the addition lines differed depending on which *Ae. biuncialis* chromosome pair was added to wheat (Fig. 1).

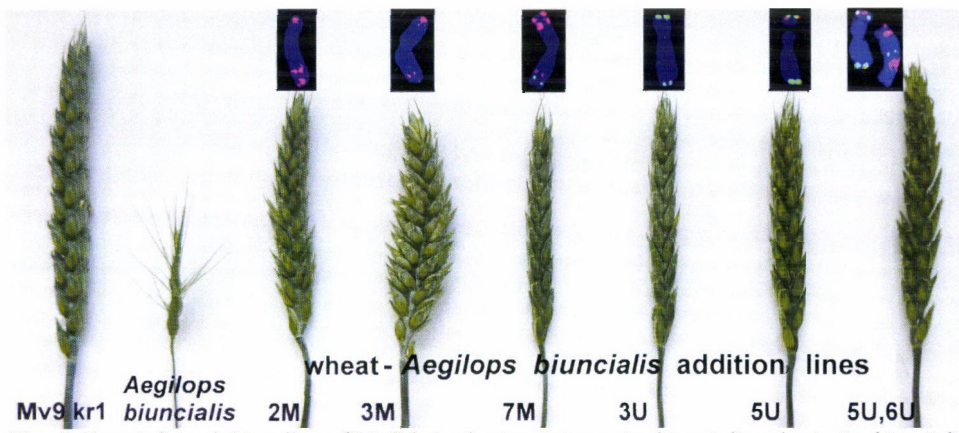

Fig. 1. From left to right, spikes of Mv9 kr1 wheat genotype, *Ae. biuncialis*, wheat–*Ae. biuncialis* disomic addition lines $2M^b$, $3M^b$, $7M^b$, $3U^b$, $5U^b$ and $5U^b/6U^b$. The FISH pattern of one of the pair of *Ae. biuncialis* chromosomes in the addition line, obtained with the repetitive DNA probes pSc119.2 (green) and pAs1 (red), is shown above each spike

In this study 119 wheat SSR markers were tested on the wheat line Mv9 kr1 and on *Ae. biuncialis*. It was found that 50.42% of the wheat microsatellite markers tested gave polymorphic or non-polymorphic PCR products on *Ae. biuncialis*. Forty-nine of the 119 markers (41.17%) did not exhibit polymorphism between Mv9 kr1 and *Ae. biuncialis*. For 30 of the markers (25.21%) bands were obtained on wheat line Mv9 kr1, while no PCR product was observed on *Ae. biuncialis*. A further 30 SSR markers (25.21%) proved to be polymorphic, i.e. PCR products were obtained on both Mv9 kr1 and *Ae. biuncialis*, but the fragment lengths differed (Fig. 2). The polymorphic markers

that also gave products on *Ae. biuncialis* were tested on the wheat–*Ae. biuncialis* addition lines $2M^b$, $3M^b$, $7M^b$, $3U^b$ and $5U^b$ (Logojan and Molnár-Láng, 2000; Molnár-Láng et al., 2002; Schneider et al., 2005), and on the $1U^g$, $2U^g$, $3U^g$, $4U^g$, $5U^g$, $7U^g$, $1M^g$, $2M^g$, $4M^g$, $5M^g$, $6M^g$ and $7M^g$ addition lines of wheat–*Ae. geniculata* (Friebe et al., 1999). Of the 30 polymorphic primer pairs that gave products on *Ae. biuncialis*, three (10%) proved to be chromosome-specific on the wheat–*Ae. biuncialis* or wheat–*Ae. geniculata* addition lines.

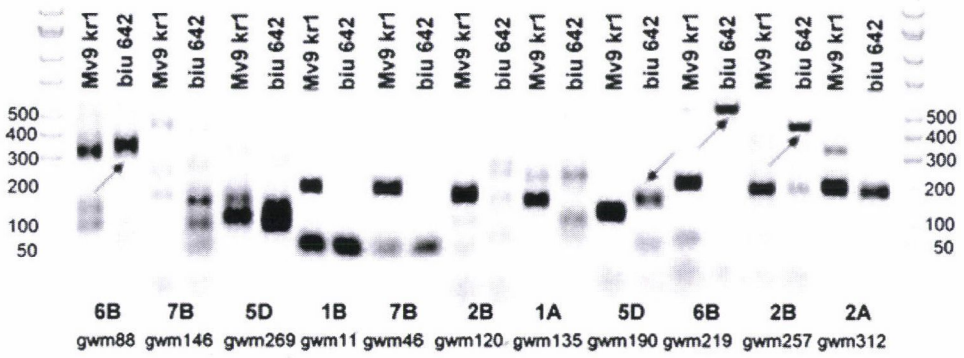

Fig. 2. Band patterns obtained for the wheat SSR markers *Xgwm88, Xgwm146, Xgwm269, Xgwm11, Xgwm46, Xgwm120, Xgwm135, Xgwm190, Xgwm219, Xgwm257* and *Xgwm312* on wheat line Mv9 kr1 and on the *Ae. biuncialis* gene bank accession MvGB642 (biu 642). Bands specific to *Ae. biuncialis* and *Ae. geniculata* are indicated by arrows. Among the markers seen to give polymorphic bands on *Ae. biuncialis* (*Xgwm88, Xgwm190, Xgwm219, Xgwm257*), none exhibited chromosome specificity on *Ae. biuncialis* or *Ae. geniculata* addition lines

Discussion

The aim of this study was to produce and identify wheat–*Ae. biuncialis* addition lines. Detailed FISH analysis was performed on various wheat, *Ae. umbellulata*, *Ae. comosa* and *Ae. biuncialis* accessions using two repetitive DNA probes (pSc119.2 and pAs1) to facilitate the identification of the *Ae. biuncialis* chromosomes in the addition lines. The level of FISH polymorphism was higher in the M^b genome than in the U^b genome. The theory of pivotal-differential genomes in *Aegilops* species was suggested by Zohary and Feldman (1962). In *Ae. biuncialis* the U genome is the pivot (stable genome), while M genome is differential. This observation was confirmed in later studies on chromosome pairing in wild wheats and hybrids (Feldman, 1965), with STS markers (Chee et al., 1995) and AFLP analysis (Monte et al., 2001). The development of wheat–*Ae. biuncialis* addition lines allows the study of the genetic effects of individual chromosomes added to the wheat genome, the tracing of *Ae. biuncialis* chromosomes in the translocation lines produced and the determination of the chromosomal location of any resistance genes transferred from *Ae. biuncialis* into wheat.

In the course of this study, three SSR markers specific to the U or M genome were selected from 119 wheat microsatellite markers. Wheat microsatellite (SSR) markers appear to be suitable for the development of SSR markers specific to the U and M genomes of *Aegilops* species, as 50.42% of the wheat microsatellite markers gave amplification products on *Ae. biuncialis*. Other authors have studied the transferability of wheat microsatellite markers to diploid *Aegilops* species. Lelley et al. (2000) and Adonina et al. (2005) observed that a large percentage of the microsatellite markers specific to the D and B genomes of wheat amplified PCR products on *Aegilops* species carrying D or S genomes. The high transferability of wheat microsatellite markers to the genome of *Aegilops* species confirms the close relationship between cultivated wheat and the *Aegilops* species. The homoeology between the chromosomes of wheat and various *Aegilops* species has also been examined using molecular markers and other molecular cytogenetic methods (Fernandez-Calvin and Orellana, 1992; Zhang et al., 1998).

The development of SSR markers specific to the U and M genomes will facilitate further cluster analysis on *Aegilops* species carrying these genomes, thus allowing chromosome fragments from these genomes to be traced more accurately in introgression lines developed using these species.

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INVESTIGATIONS ON THE REGENERATION ABILITY OF SQUASH CULTIVARS

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Pumpkin, squash and zucchini are important vegetable crops in tropical and temperate regions. The development of genetic transformation methods offers the potential of introducing valuable traits into these crops. An efficient *in vitro* plant regeneration system is a critical point for genetic manipulation. The regeneration ability of three *Cucurbita* varieties was tested on Murashige and Skoog medium supplemented with different growth regulators. Cotyledons of all the varieties were cultured to investigate the effect of 2,4-D (0, 1, 2, 3, 4 mg l^{-1}) with or without KIN (0, 0.5, 5 mg l^{-1}) and of BA (0, 1, 1.2 mg l^{-1}) combined with IAA (0, 0.9, 1, 1.2 mg l^{-1}), on the efficiency of shoot induction. Abscisic acid (0.26 mg l^{-1} ABA) was also added to one medium. To find the most suitable combination for shoot induction, cotyledon segments of the three varieties were also cultivated on media with different concentrations of BA (0–1.2 mg l^{-1}) and IAA (0–0.9 mg l^{-1}). Shoot induction was achieved via organogenesis in the tested varieties. Leafy shoots were transferred to root induction media. Regenerated plantlets with roots were transferred to sterile soil. This is the first report on *in vitro* regeneration from cotyledon explants of the pumpkin cultivar Nagydobosi and the pattypan squash cultivar Óvári fehér.

Key words: *in vitro*, regeneration, organogenesis, cotyledon, squash, zucchini, pumpkin

Abbreviations: 2,4-D – 2,4 dichlorophenoxyacetic acid, ABA – abscisic acid, BA – 6-benzylaminopurine, IAA – indole-3-acetic acid, KIN – kinetin, NAA – 1-naphthalene acetic acid

Introduction

Cucurbita pepo L. comprises a diverse range of crops including pumpkins, winter squash and various kinds of summer squash, including zucchini and pattypan squash. *Cucurbita* genus is considered to be a major vegetable crop in tropical and temperate regions. Worldwide production of *Cucurbita* reached 21 million metric tonnes in 2007 (FAOSTAT, 2009).

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However, *Cucurbita* is noted to have been affected by several viral diseases. Plant biotechnology appears to be a viable option for the improvement of the *Cucurbita* species by means of plant tissue culture and genetic transformation. The establishment of an efficient *in vitro* plant regeneration system suitable for genetic transformation is a key step in this approach (Zhang et al., 2008). The development of genetic transformation for squash offers the potential of introducing valuable traits, e.g. disease resistance, high sugar content and high protein content, to improve productivity and quality beyond the limits of conventional breeding.

Regeneration in the *Cucurbita* genus has been reported in earlier studies. Schroeder (1968) regenerated zucchini (*Cucurbita pepo*) via somatic embryogenesis from fleshy pericarp wall-derived callus. Jelaska (1972; 1974) reported somatic embryogenesis in hypocotyl- and cotyledon-derived callus of pumpkin (*C. pepo*) and demonstrated that embryos could develop into normal plants. In most of the experiments the explant source was cotyledons (Jelaska, 1972; Katavic et al., 1991; Chee, 1992; Gonsalves et al., 1995; Abrie and van Staden, 2001; Lee et al., 2003; Urbanek et al., 2004) or cotyledon explants with hypocotyl segments (Ananthakrishnan et al., 2003; 2007; Kathiravan et al., 2006). Explants from hypocotyls (Jelaska, 1974; Jelaska et al., 1985; Lee et al., 2002), leaves (Kintzios et al., 2002), roots (Lee et al., 2002), mechanically wounded mature embryos (Leljak-Levanic et al., 2004), seedling-derived shoot tip segments (Shah et al., 2008), single nodes (Juretic et al., 1989), internode segments (Rahman et al., 1993), unpollinated ovules (Kwack and Fujieda, 1988; Metwally et al. 1998a) and anther culture (Metwally et al., 1998b) were also used in some experiments. In the recent work of Amutha et al. (2009a) adventitious shoot regeneration was induced using greenhouse-grown seedlings in the cotyledon stage by decapitation, leaving a single cotyledon after removing the apical meristem.

Most authors reported regeneration via somatic embryogenesis (Jelaska, 1972; 1974; Chee, 1991; 1992; Gonsalves et al., 1995; Kintzios et al., 2002; Urbanek et al., 2004), but regeneration has also been reported via direct shoot organogenesis (Ananthakrishnan et al., 2003; Kathiravan et al., 2006) in *Cucurbita pepo*. The purpose of this study was to establish a reliable system to regenerate three different varieties of *Cucurbita pepo* L. The varieties tested all had special value in some respect.

Materials and methods

Plant material

The regeneration ability of three *Cucurbita* varieties: pattypan squash (*C. pepo* convar. *Patissoniana*) Óvári fehér, zucchini (*C. pepo* convar. *Giromontiina*) Black Beauty and pumpkin (*C. maxima* convar. *Maxima*) Nagydobosi, also known as Hungarian Mammoth, was tested using seeds provided by the Genebank of the Corvinus University of Budapest (Budapest, Hungary).

After manual removal of the seed coats, the seeds were surface-sterilized for 30 minutes in 15% Clorox and washed three times with sterile distilled water. The seeds were sown on MS (Murashige and Skoog, 1962) medium. For germination they were kept at 32°C in darkness for two days, followed by two days in a growth chamber at 25°C, with a 16 h/8 h light/dark photoperiod provided by cool-white fluorescent lamps.

Explant preparation

After removing the major part of the tip and the petiole, the cotyledons were halved transversely and their basal part was used to give 2 explants per seedling. Three explants were placed in each glass vessel (200 ml, diameter 5 cm) with the abaxial side down. Seven- to ten-day-old green, fully-expanded cotyledons were used in the experiments.

Culture media and conditions

Cotyledon segments were cultivated on MS medium supplemented with different combinations of growth regulators. In the first series of experiments seven different media were tested. Cotyledons of all the varieties were cultured to investigate the effect of 2,4-dichlorophenoxyacetic acid (0, 1, 2, 3, 4 mg l^{-1}) with or without kinetin (0, 0.5, 5 mg l^{-1}) and of 6-benzylaminopurine (0, 1, 1.2 mg l^{-1}) combined with indole-3-acetic acid (0, 0.9, 1, 1.2 mg l^{-1}) on the efficiency of shoot induction. Abscisic acid (0.26 mg l^{-1}) was also added to one medium containing 1 mg l^{-1} BA + 0.9 mg l^{-1} IAA. To find the most suitable medium for shoot induction, cotyledon segments of the three varieties were also cultivated on media with different concentrations of BA (0, 0.6, 0.8, 1, 1.2 mg l^{-1}) and IAA (0, 0.1, 0.3 0.6, 0.9 mg l^{-1}). All the media were supplemented with 3% sucrose, MS vitamins and 2.5 g/l Phytagel (Duchefa) and the pH was adjusted to 5.6–5.8 before autoclaving (120°C, 20 min). Three explants were placed in each glass vessel (200 ml). Three independent experiments were conducted for data analysis. Each treatment consisted of nine cotyledon explants. After 4–5 weeks of culture the regenerating areas (multiple shoots) of the explants were excised and transferred to fresh regeneration medium. After a further 5 weeks of culture the explants were scored, with the aid of a stereomicroscope, for the number of explants forming shoot buds or leafy shoots.

Root induction and plant acclimatization

For root induction, excised shoots which had at least 5–6 fully opened leaves were transferred to growth regulator-free MS medium solidified with 8 g l^{-1} agar, and grown until they had a well-developed root system. The plantlets were acclimatized in soil/vermiculite mixture (1:1) and incubated at 25±1°C in a growth chamber for 2 weeks at 100% relative humidity and for another 2 weeks with gradually decreasing humidity. After these steps they were transferred to a greenhouse.

Results

In the first series of experiments cotyledons of all the varieties were cultured on four different media containing 2,4-D with or without kinetin. The explants enlarged and became green, but none of the varieties showed shoot regeneration. Only white, translucent callus was formed, and root induction was also observed (data not shown). On medium containing BA and IAA with or without ABA a mixture of leaf and bud primordia appeared on the cotyledon segments of all the varieties within 1–2 weeks and formed multiple buds. From these multiple shoots, usually only one shoot developed on each cotyledon segment. To evaluate the final results only leafy shoots with 3 or 4 leaves showing the normal phenotype were counted. The highest number of plantlets was obtained on explants of all the varieties on the medium containing 1 mg l^{-1} BA + 1.2 mg l^{-1} IAA (Table 1).

Table 1
Shoot regeneration frequency on cotyledon explants of Óvári fehér, Black Beauty and Nagydobosi on media with different growth regulators

Growth regulators (mg l^{-1})			No. of shoots per explant		
IAA	BA	ABA	Óvári fehér	Black Beauty	Nagydobosi
0.9	1	0.26	0.07±0.06	0.29±0.06	0.22±0.11
1	1.2	0	0.11±0.11	0.29±0.12	0.26±0.12
1.2	1	0	0.14±0.02	0.37±0.16	0.29±0.06

Each value represents the mean±SD of nine explants in three repeated experiments

In the second series of experiments, 25 different growth regulator combinations were tested. After a few days of culture on regeneration medium, green callus formation was observed for all the varieties. After two weeks, multiple shoot buds were formed on most of the cotyledon explants. Some exhibited small clusters of shoot initials and after 3–4 weeks started to form callus. Others formed leaf primordia, but no shoots developed from them. The best results were obtained on media supplemented with BA in the range of 0.6–1 mg l^{-1} combined with 0–0.6 mg l^{-1} IAA. Some of the regenerated shoots were malformed, but these were not counted in the final results.

Among the varieties tested, the pumpkin variety Nagydobosi gave the best regeneration response. On almost every growth regulator combination, multiple shoots were obtained (92%) after 9 days (Fig. 4A). The highest number of well-formed plantlets (ready to transfer to rooting media) was counted on medium supplemented with 1 mg l^{-1} BA + 0.1 mg l^{-1} IAA. On this medium 0.53 plants were regenerated per explant. Increasing the concentration of BA resulted in a higher number of shoots and the best results were achieved on media containing 1 mg l^{-1} BA, but a further increase in the concentration decreased the number of shoots (Fig. 1). The regeneration of the zucchini variety Black Beauty showed better results on medium containing only 0.6 mg l^{-1} BA (0.67 shoots/explant). Regeneration was observed on 76% of the media with the tested growth regulator combinations (Fig. 2). In the case of the pattypan squash variety Óvári fehér only 56% of the tested growth regulator combinations produced shoot initials from cotyledonary segments. The best regeneration response was observed on medium containing 0.8 mg l^{-1} BA (0.33 shoots/explant). Increasing the concentration of BA resulted in a higher number of shoots, but increasing that of IAA decreased the number of shoots (Fig. 3).

Regenerated shoots with 5 or 6 fully opened leaves were excised from the explants for *in vitro* root induction (Fig. 4B). On regulator-free MS medium adventitious roots developed after 10–14 days of culture (Fig. 4C). In the case of zucchini, root induction often took place on the regeneration medium. The rooted shoots were successfully acclimatized in an environment-controlled growth chamber; the plants were normal and produced flowers in the greenhouse.

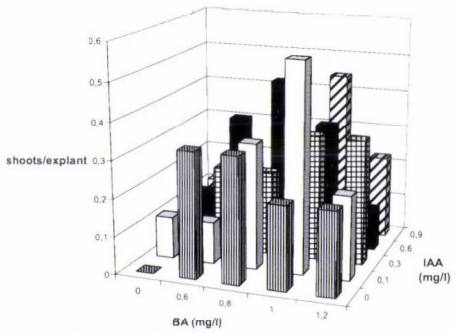

Fig. 1. Effect of growth regulator combinations on the pumpkin variety Nagydobosi

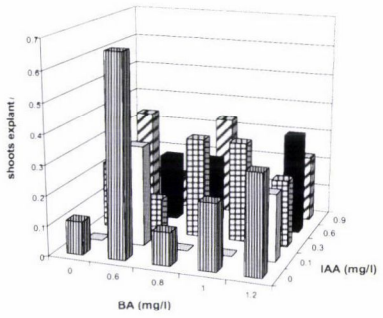

Fig. 2. Effect of growth regulator combinations on the zucchini variety Black Beauty

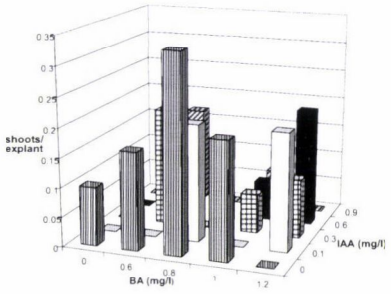

Fig. 3. Effect of growth regulator combinations on the pattypan squash variety Óvári fehér

Discussion

When the regeneration ability of three squash cultivars was tested, considerable differences were observed, with better regeneration efficiency for Nagydobosi and Black Beauty than for Óvári fehér. The results obtained with seven different media showed that explants of each variety regenerated on similar combinations of growth regulators, but the regeneration responses were different. In contradiction to the findings of Gonsalves et al. (1995), where 2,4-D alone was able to induce embryogenesis, in the present experiments the explants showed no regeneration response on medium containing only 2,4-D. Combining 2,4-D with kinetin had no positive effect on the results. On medium containing BA and IAA with or without ABA multiple buds appeared on cotyledon segments of the three varieties, and for all the varieties the highest number of plantlets was observed on the medium containing 1 mg l^{-1} BA +1.2 mg l^{-1} IAA. In the study of Abrie and van Staden (2001) no shoot regeneration occurred when BA and IAA combinations were used for three different *Cucurbita* cultivars. This confirms that genotype is a determining factor in the regeneration of Cucurbitaceae.

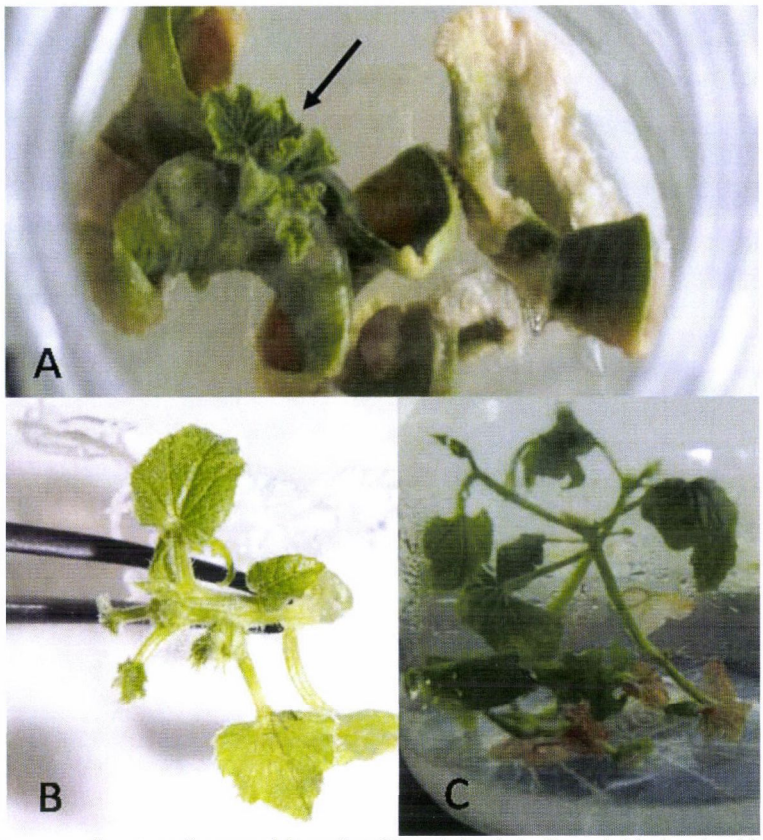

Fig. 4. Regeneration steps for pumpkin variety Nagydobosi. A: Shoot initiation (multiple shoot cluster) after 9 days; B: Regenerated plantlet with 5–6 leaves, just before transferring to rooting medium; C: Pumpkin plant with roots

In the experiments on growth regulator combinations various concentrations of BA alone induced regeneration in all the varieties, and in the case of pattypan squash Óvári fehér and zucchini Black Beauty the best results were achieved on these media (Figs. 2 and 3). A similar effect of BA (1 mg l^{-1}) was reported by other authors (Ananthakrishnan et al., 2003; 2007; Lee et al., 2003; Kathiravan et al., 2006; Amutha et al., 2009b). In the present experiments there was no significant difference between the BA concentrations, similarly to the work of Zhang et al. (2008), who also reported no significant difference between the number of shoots per explant on media supplemented with 0.5, 1 or 2 mg l^{-1} BA. In the case of the pumpkin variety Nagydobosi, shoot regeneration was more efficient when BA was applied together with IAA (Fig. 1). This confirms the results of Urbanek et al. (2004), where oilseed pumpkin (*Cucurbita pepo* var. *styriaca* Greb.) plants were regenerated from cotyledon explants via somatic embryogenesis on media supplemented with cytokinin (BA) and auxin (NAA). The results of the present regeneration experiments are in agreement with previous observations (Jelaska, 1972; Katavic et al., 1991; Chee, 1992;

Gonsalves et al., 1995; Abrie and van Staden, 2001; Lee et al., 2003; Urbanek et al., 2004; Zhang et al., 2008) that cotyledons are a suitable explant source and that in the case of winter squash (*Cucurbita maxima* Duch.) the basal part of halved cotyledons is even more responsive in regeneration experiments (Lee et al., 2003). Kathiravan et al. (2006) obtained 1.2–3.9 shoots per cotyledon segment in a study on 15 different squash varieties.

It is generally known that responsiveness is extremely genotype-dependent, so the present results can be regarded as novel. Shoot formation was primarily obtained on wounded surfaces or the surrounding tissues, which benefits *Agrobacterium*-mediated transformation. This is the first report on *in vitro* regeneration from cotyledon explants of the pumpkin variety Nagydobosi and the pattypan squash variety Óvári fehér.

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GENERAL AND SPECIFIC COMBINING ABILITY OF *in vitro* DOUBLED HAPLOID MAIZE LINES IN THE FIELD

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The breeding of hybrid maize now has a history of over 100 years. In 1908, George H. Shull was the first to report on the high yields, great uniformity and homogeneity of hybrids derived from a cross between two inbred lines. Following this discovery, consistent self-fertilisation over a period of six to eight generations was found to be an extremely efficient method for developing maize lines. From the mid-1970s, however, with the elaboration of the monoploid (*in vivo*) and microspore culture *(in vitro*) techniques, it became possible to develop homozygous lines within a year.

With the help of an efficient plant regeneration system based on anther culture, large numbers of doubled haploid (DH) lines can be produced. In the course of the experiments the seed of DH plants selected over several years was multiplied and crossed with Martonvásár testers, after which the hybrids were included in field performance trials in three consecutive years (2005–2007). The aim was to determine whether the field performance of hybrids developed in this way equalled the mean yield of standards with commercial value. The data also made it possible to calculate the general (GCA) and specific (SCA) combining ability of the parental lines, indicating the usefulness of the parental components in hybrid combinations and expressing the extent to which a given line contributes to yield surpluses in its progeny.

A total of 52 maize hybrids were evaluated in the experiments in terms of yield and grain moisture content at harvest. The combinations, resulting from crosses between 12 DH lines, one control line developed by conventional inbreeding and four testers, were found to include hybrids capable of equalling the performance of the standards, and four DH lines were identified as improving the yield level of their progeny. As the experiment was carried out on a very small number of genotypes, the results are extremely promising and suggest that, if the range of genotypes used to develop DH lines is broadened and the sample number is increased, it will be possible in the future to find maize hybrids, developed with *in vitro* DH parental components, that surpass the performance of commercial hybrids.

Key words: maize lines, doubled haploid, combining ability, GCA, SCA, performance trials

Introduction

The history of hybrid maize breeding began over 100 years ago. George H. Shull was the first to report on the high yields, great uniformity and homogeneity of hybrids derived from a cross between two inbred lines (Shull, 1908). Six years later he introduced the idea of heterosis, which he referred to as hybrid vigour (Shull, 1914). By the 1930s the cultivation of open-pollinated maize varieties was largely replaced by that of inbred hybrids. The maize hybrids developed using the new breeding method surpassed the local varieties previously grown mainly in terms of yield and uniformity (the latter became particularly important with the spread of mechanisation in agriculture). Inbreeding became a basic tool in conventional maize breeding, allowing the development of breeding materials that could be maintained in stable form for long periods, were clearly distinguishable from other lines, and had a high level of uniformity.

The time required for self-fertilisation was 6–8 generations, accompanied by continual selection. The last phase of the work process was the test crossing of the inbred lines to determine the extent to which the value of the parents was manifested in the hybrids. If the appearance and resistance of the lines and the performance and yield stability of the hybrids suited the purpose of the breeder, a new inbred line was born, which, depending on its value, could play a role in maize production in Hungary, Europe, or even in other parts of the world, for a longer or shorter period.

Maize anther culture was first developed in the early seventies when a Chinese team (Research Group 401) produced callus from pollen and used growth regulators to regenerate haploid plants, the pollen origin of which was proved by chromosome analysis. In the early years MS medium (Murashige and Skoog, 1962) was used for anther culture, which was later supplemented with auxins (primarily 2,4-D). The real breakthrough came with the development of N_6 medium by Chu et al. (1975), various modifications of which were widely used. Later this was the medium composition generally employed for maize tissue culture.

Research on the artificial rediploidisation of the genome began in 1974, and colchicine proved to be the best agent for achieving the doubling of the haploid chromosome set (Jensen, 1974). Wan et al. (1989) placed chopped calli of anther origin on filter paper in liquid D nutrient medium (Duncan et al., 1985) containing 0.025% or 0.05% colchicine and incubated them for 24, 48 or 72 hours. After 72 hours of treatment with 0.025% or 0.05% colchicine almost 100% of the haploid calli exhibited reduplication, while this figure was only 50% after 24-h treatment. However, spontaneous doubled haploids may also occur in maize anther cultures. Dieu and Beckert (1986) reported a spontaneous rediploidisation ratio as high as 22%.

Studies on the genetic regulation of *in vitro* androgenesis revealed additive genetic interactions in maize (Petolino and Thompson, 1987). However, the trait was also substantially influenced by environmental and additive genetic variance (Wan et al., 1991). Due to the decisively additive nature of the trait, it is relatively simple to introduce haploid induction ability into genotypes with a poor response to tissue culture, using both conventional and *in vitro* techniques. This ability can be transferred from one genotype to the other by simple backcrossing (Obert et al., 1998).

Orosz and Barnabás (1997) crossed eight DH lines of Chinese origin with five elite inbred lines from Martonvásár in order to analyse the agronomic properties of the DH lines, elite inbred lines and their hybrids in a two-year field experiment. The DH lines characteristically exhibited multiple ears, a trait reliably inherited by their progeny. The elite parents caused the hybrids to flower earlier than the DH parents. The inbred lines flowered 77–78 days after sowing, on average. This figure was 86–88 days for the DH lines and 78–79 days for the hybrids.

Szundy et al. (1995) crossed derivatives of two DH lines of Chinese origin with elite inbred lines from Martonvásár (as testers) and studied the hybrid combinations in small-plot field experiments. The tested DH lines originated from DH 109 (Chi 592) and DH 105 (Chi 592×A_2). The testers belonged to the Stiff Stalk Synthetic and Lancaster groups, while one differed from these and could not be assigned to any group. The following characteristics were analysed in the test crosses: plant height, ear attachment height, number of days to 50% silking and 50% tasselling. In addition the following yield components were recorded: ear length, number of kernel rows and thousand-kernel mass. The flowering period was found to range from 67–84 days, the plant height from 202–277 cm, the ear attachment height from 75–146 cm, the ear length from 17.4–20.0 cm, the number of kernel rows from 13–16 and the thousand-kernel mass from 291–399 g. Some of the DH lines possessed good disease resistance or stalk strength and some appeared to have good combining ability, so it was concluded that these could be promising breeding sources in the future.

Inbred lines can be evaluated from several points of view, based on their external appearance, morphological data, chemical quality, abiotic and biotic stress tolerance, maturity group, origin, etc. Their characteristics depend largely on the decisions of the breeder, which play an important role in how the inbred line is judged. However, there is a need for parameters which express the extent to which the usefulness of the line is manifested in the progeny. The values of such parameters indicate the mean improvement that individual inbred lines induce in the performance of their hybrid progeny.

The term progeny testing was defined in the 1960s as 'a test of the value of a genotype based on the performance of its offspring produced in some definite system of mating' (Allard, 1960). Hopkins was the first to carry out progeny tests on maize in 1896, in an attempt to increase the oil and protein content (Hallauer and Miranda, 1981).

The combining ability of inbred lines is a factor that determines the usefulness of the lines in hybrid combinations. The value of the line can best be expressed through the performance of crossing combinations (Hallauer and Miranda, 1981).

The terms general (GCA) and specific (SCA) combining ability were introduced by Sprague and Tatum (1942). In the original sense, the GCA can be determined by using a broad base heterogeneous population as tester, while differences in the SCA can be revealed using a tester with a narrow genetic base (inbred line or single cross).

The GCA thus expresses the mean performance of a parental line in hybrid combinations, while the SCA is a measure of the value of individual combinations as a function of the mean performance of the parental components. GCA and SCA are always relative values and depend greatly on the performance of the inbred lines involved in the combinations. The value of GCA tends to express additive gene effects, while SCA is more indicative of dominant and epistatic effects.

Materials and methods

The haploid tissue culture was based on an exotic line of Chinese origin with excellent anther induction ability (Chi 592), obtained in the framework of research cooperation. The genotype DH 109, a direct DH descendant of this line, was obtained by multiple *per se* selection in the Cell Biology Department of the Martonvásár institute, and contains the gene responsible for successful tissue culturing. Another nearly-related line, DH 105, was also used. This originated from a cross between DH 109 and SR 88, a line resistant to sulphonylurea, and was also developed using the haploid tissue culture technique, followed by selection.

An elite Martonvásár line with a poor response to tissue culture (Mv Line) was also included in the experiment. This extremely valuable line, the parental component of many commercial hybrids, was developed in Martonvásár in the mid-1990s and is of Iodent origin. Its breeding value is clear from the fact that it is still used in the development of numerous commercial hybrids.

Twelve DH maize lines and the elite Martonvásár line were used as parental components in crossing experiments in Martonvásár in three consecutive years (2005–2007). These DH lines were developed previously from combinations of Chinese lines with good haploid induction ability and Martonvásár inbred lines used in commercial hybrids. The inbred lines belonged primarily to the Iodent group (10 DH lines), with two from the Mindszentpusztai Yellow Dent group (DH 53 and DH 63). The DH lines were developed in F_1 and BC_1 combinations so that the commercial line was present in various proportions.

Three testers were developed from sister line crosses of Iodent (Iodent SLC), Lancaster (Lanc. SLC) and Iowa Stiff Stalk Synthetic (ISSS SLC), while the fourth was a non-related tester belonging to none of these groups (NR SLC). All four testers are the parental components of hybrid combinations with commercial value, still on the market.

The performance of the hybrids was compared with that of genotypes developed by breeding companies in Hungary and used as FAO 390 and FAO 450 standards in the 2005–2006 official state trials.

The hybrid experiments were laid out in two-row plots 5.6 m in length, with row and plant spacings of 72 and 20 cm, giving a total plant number of 56 in each replication (equivalent to a plant density of 70,000 plants ha^{-1}).

Measurements were made using a grain moisture content meter attached to the combine (based on the electrical conductance principle) and a small-plot balance. The grain yield was converted to a uniform moisture content of 14%.

The Martonvásár experiments were set up according to the factorial pairing model, in which the 12 DH lines and the control Mv Line were used as male parent and crossed with the four testers as female parent, giving a total of 52 hybrids. As the FAO 390 and FAO 450 standards were also sown, 54 hybrids were examined each year in a performance trial with three replications.

The statistical analysis was carried out using the "Agrobase 99® for Microsoft Windows®" computer software (Agronomix Inc.) and evaluated following the guidelines of Sváb (1981). The data were compiled using the MS Excel program.

Results

The performance of the hybrids in the factorial pairing model experiment was divided into two main components, which were measured at harvest. One was the grain yield per plot, converted to yield per hectare (t ha^{-1}) for a grain moisture content of 14%, while the other was the grain moisture content (%) at harvest (Tables 1 and 2; Fig. 1).

It can be seen from the GCA values that four of the 12 DH lines resulted in yield surpluses in the hybrids (DH 56: 0.41 t ha^{-1}; DH 136: 0.38 t ha^{-1}; DH 57: 0.30 t ha^{-1}; DH 143: 0.10 t ha^{-1}). The control (Mv Line) had an outstanding yield-increasing effect, with an average increase of 1.45 t ha^{-1} in the hybrids. Among the testers, the combining ability of the non-related tester had the best effect on the performance of the hybrids (0.49 t ha^{-1}).

Table 1

General and specific combining ability (t ha^{-1}) of the parental components, calculated from the grain yield of the hybrids (2005–2007)

Genotypes	Genetic background	Iodent SLC	Lanc. SLC	ISSS SLC	NR SLC	GCA
		SCA				
DH 109	Chi 592	1.09	–0.20	–0.61	–0.08	–0.53
DH 384	Chi 592×A2	0.54	–0.19	–0.40	0.28	–0.51
DH 136	DH 109×Mv Line	0.02	0.11	**0.60**	–0.52	**0.38**
DH 143	(DH 109×Mv Line) × Mv Line	–0.27	0.19	0.01	**0.28**	**0.10**
DH 31	Mv Line×DH 109	0.83	0.34	0.32	–1.26	–0.21
DH 141	(Mv Line×DH 109) × Mv Line	0.34	1.09	–1.33	0.10	–0.07
DH 105	SR 88×Chi 592	0.14	–0.21	–0.16	0.45	–0.21
DH 57	DH 105×Mv Line	–0.44	**0.87**	0.26	–0.48	0.30
DH 64	DH 105×Mv Line	–1.00	–0.43	1.29	0.36	–0.19
DH 56	(DH 105×Mv Line) × Mv Line	–0.67	–0.98	**1.09**	**0.77**	**0.41**
DH 53	HMv 651-4-ET3 × DH 105	0.38	–0.81	0.48	0.17	–0.03
DH 63	DH 104×(HMv 5302 × HMv 124-2)	0.00	0.31	–0.22	0.15	–1.58
Mv Line	Mv Line	–0.22	**0.59**	–0.68	**0.53**	**1.45**
GCA		–0.19	–0.28	–0.24	**0.49**	

Data satisfying the criteria are printed in bold.

Table 2
General and specific combining ability (%) of the parental components, calculated from the grain moisture content at harvest of the hybrids (2005–2007)

Genotypes	Genetic background	Iodent SLC	Lanc. SLC	ISSS SLC	NR SLC	GCA
		SCA				
DH 109	Chi 592	–1.63	–0.08	0.59	0.90	1.83
DH 384	Chi 592×A2	**–0.40**	1.63	**–0.50**	–0.97	**–0.05**
DH 136	DH 109×Mv Line	–1.00	–1.01	0.97	0.82	0.84
DH 143	(DH 109×Mv Line) × Mv Line	0.21	–1.22	0.35	0.44	0.35
DH 31	Mv Line×DH 109	–0.59	0.85	–1.01	0.51	2.19
DH 141	(Mv Line×DH 109) × Mv Line	**–0.36**	–1.32	1.48	–0.02	**–0.20**
DH 105	SR 88×Chi 592	–0.49	1.26	–0.76	–0.21	0.02
DH 57	DH 105×Mv Line	–0.20	1.30	–0.71	–0.62	0.39
DH 64	DH 105×Mv Line	–0.18	–0.59	0.46	0.09	**–0.55**
DH 56	(DH 105×Mv Line) × Mv Line	0.02	–0.33	0.03	0.07	**–0.97**
DH 53	HMv 651-4-ET3 × DH 105	**–0.18**	0.65	**–0.05**	–0.64	**–0.08**
DH 63	DH 104×(HMv 5302 × HMv 124-2)	0.75	–0.50	**–0.09**	–0.38	**–2.81**
Mv Line	Mv Line	3.38	–1.34	**–1.52**	–0.74	**–0.25**
GCA		**–1.35**	0.83	**–0.03**	0.77	

Data satisfying the criteria are printed in bold.

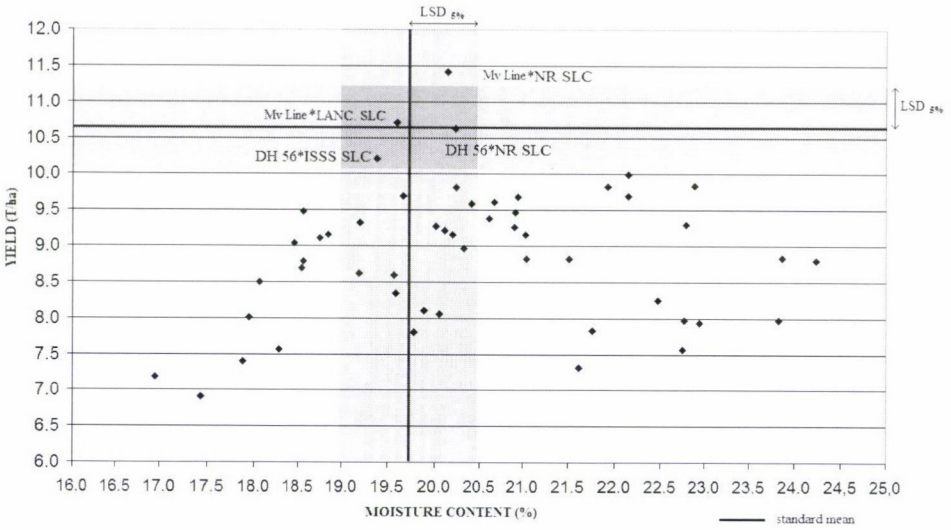

Fig. 1. Diagram of the yield and grain moisture content at harvest of hybrids with DH parental components (Martonvásár, 2005–2007)

SCA values are more difficult to interpret, as the results also depend on the GCA values (improving effect) of the parents. The following combinations were found to result in a further yield increase, in addition to the yield-improving effect of the parental lines: DH 56 × NR SLC (0.77 t ha^{-1}); Mv Line × NR SLC (0.53 t ha^{-1}) and DH 143 × NR SLC (0.28 t ha^{-1}).

Four combinations were found where a relatively high, positive SCA value was combined with a yield-improving effect from at least one of the parents. These were DH 56 × ISSS SLC (1.09 t ha^{-1}), DH 57 × Lanc SLC (0.87 t ha^{-1}), DH 136 × ISSS SLC (0.60 t ha^{-1}) and Mv Line × Lanc SLC (0.59 t ha^{-1}).

The GCA and SCA values calculated for each line from the grain moisture values of the hybrids are presented in Table 2. In this case, negative values are sought, as it is desirable to achieve lower grain moisture content at harvest.

Six DH lines were found to have favourable GCA values for this trait, improving the grain moisture content of the hybrids at harvest by 0.05–2.81%. This effect was most pronounced for DH 63, which was the earliest maturing line in the plant material.

Among the testers, Iodent SLC (–1.35%) and ISSS SLC (–0.03%) were found to improve the grain moisture content of the hybrids, while the other two testers resulted in a slight increase, though this may be related to the fact that the Lanc SLC and NR SLC male plants flowered later (data not shown). The control Mv Line also resulted in an improvement in the grain moisture content of the combinations (–0.25%).

Seven combinations exhibited a greater improvement in grain moisture content than could be expected from the improving effect of the parents, i.e. the SCA value was negative and the GCA values of the parents were also negative for grain moisture content. The greatest SCA value was obtained for the combination Mv Line × ISSS SLC, where the grain moisture content of the hybrid at harvest was more than 1.5% lower.

The combined values of the 52 hybrids for grain yield and grain moisture content at harvest are illustrated in Figure 1, which demonstrates that the majority of the hybrids had significantly lower yields than the mean of the standards. Nevertheless, a few combinations of DH origin were found to yield on par with the mean of commercial hybrids (DH 56 × ISSS SLC and DH 56 × NR SLC). Two combinations produced using the control line also performed well, one equalling the standard mean (Mv Line × Lanc SLC) and the other significantly exceeding it (Mv Line × NR SLC).

More than half the hybrids had grain moisture contents at harvest that were close to those recorded for the standards. However, in most cases, satisfactory grain moisture content was not associated with high grain yield, so only the most promising four combinations were analysed in more detail, none of which was significantly different from the standards. The grand mean of the experiment was 8.95 t ha^{-1}, which was significantly lower than the standard mean (10.66 t ha^{-1}; $LSD_{5\%}$ = 0.56 t ha^{-1}). The highest grain yield, which surpassed that of the standards, was given by the hybrid Mv Line × NR SLC (11.42 t ha^{-1}). Among the 48 DH line combinations only two gave results close to the desired level (DH 56 × NR SLC: 10.62 t ha^{-1} and DH 56 × ISSS SLC: 10.21 t ha^{-1}). A similar result was obtained for Mv Line × Lanc SLC: 10.71 t ha^{-1}.

The grand experimental mean for grain moisture at harvest was 20.36%, compared to the standard mean of 19.74% ($LSD_{5\%} = 0.71$), so the difference was not substantial. Among the hybrids, 18 combinations were statistically at par with the mean of the commercial hybrids, while 22 had significantly higher grain moisture content at harvest, and 12 significantly lower. Neither the lower nor the higher grain moisture content was associated with high grain yield, and four hybrids (two of them having DH parental components) were found to satisfy the criteria.

The hybrids created between DH lines and the Lancaster, ISSS and NR testers gave the best performances. All the combinations that approached the standard mean originated from parents with these backgrounds. Since Mv Line was of Iodent origin and the various DH lines were derivatives of this line, the results conformed to expectations, as hybrids between parental partners belonging to different heterosis groups gave the best yields. Averaged over the performance of the hybrids, normal distribution was observed for both grain yield and grain moisture content. The majority of the hybrids had grain yields between 8 and 10 t ha^{-1}, while the grain moisture content at harvest ranged from 18 to 21%.

Discussion

Based on GCA values, four of the 12 DH lines tested had a yield-improving effect. DH 56 resulted in an average yield surplus of 0.41 t ha^{-1} in its hybrids, while the improvement achieved with the other three lines (DH 136, DH 143, DH 57) was somewhat lower. The effect of the other eight DH parental lines, as expressed in the mean performance of their hybrid combinations, did not come up to expectations. The GCA value of Mv Line, tested as a control, exceeded that of the DH lines (1.45 t ha^{-1}) and was thus evaluated as the best combining line.

The majority of the testers tended to reduce the mean yield of the combinations in this pairing system, the only exception being NR SLC, which gave an improvement of 0.49 t ha^{-1}. It must not be forgotten, however, that GCA and SCA values are always relative, and depend greatly on the overall performance of the inbred lines used in the combinations (Sprague and Tatum, 1942). The DH lines in the present work formed the most favourable combinations with the NR SLC tester, while the Iodent, Lancaster and ISSS SLC males were unable to perform satisfactorily in the hybrid combinations.

The specific combining ability illustrates the value of individual combinations as a function of the mean performance of the parental components, based on their GCA values, so the SCA values can only be properly interpreted together with the GCA values of the parents.

In the light of the above, combinations were sought, where good SCA values were combined with a yield-improving effect of at least one parent (GCA).

The best combination in the experiment was Mv Line × NR SLC, where both parents had a yield-improving effect (additive genetic effect) in the hybrid (GCA: 1.45 and 0.49 t ha^{-1}), while the combination itself also resulted in a yield surplus (SCA: 0.53 t ha^{-1}; dominant and epistatic effects; Sprague and Tatum, 1942).

Among the progeny of the DH lines, the combinations DH 56 × NR SLC (GCA: 0.41 and 0.49 t ha^{-1}, SCA: 0.77 t ha^{-1}) and DH 143 × NR SLC (GCA: 0.28 and 0.49 t ha^{-1}; SCA: 0.28 t ha^{-1}) had yield-improving values of both GCA and SCA.

Combinations in which only one parent improved the performance of the hybrids are not listed here (for more details, see Tables 1 and 2). It can be seen from the results that, despite the very small number of DH lines, it nevertheless proved possible to find genotypes that improved the performance of the progeny, though this improving effect did not reach the value of the control, elite line.

Based on GCA and SCA values calculated from grain moisture contents at harvest, six DH lines (and the control Mv Line) had GCA values that met the desired criteria. These included DH 56, which resulted in an improvement of 1%. Among the testers, Iodent SLC and ISSS SLC improved the performance of the hybrids. The SCA only improved the grain moisture content of the hybrid, over and above the improving effect of the parents, in seven cases. The line DH 143, which improved the grain yield, had an unfavourable effect on the grain moisture at harvest (GCA: 0.35%), and the hybrid DH 143 × NR SLC, which performed well for grain yield, had a poor SCA value, resulting in an increase in grain moisture content of 0.44% in the harvested grains.

Only line DH 56 was found to be satisfactory for both traits. However, the performance of the hybrids can be illustrated better on the basis of yield and grain moisture at harvest, so the GCA and SCA values were complemented by the analysis of progeny performance.

The diagram depicting both the yield and the grain moisture at harvest of the hybrids exhibited normal distribution for both traits. With respect to grain yield, the majority of hybrids with DH parental components gave a significantly lower yield than the standard mean (48 combinations). Three hybrids reached the desired yield level (DH 56 × NR SLC, DH 56 × ISSS SLC, Mv Line × Lanc SLC), while one control combination (Mv Line × NR SLC) gave a significantly better yield.

The experimental grand mean (8.95 t ha^{-1}) was significantly lower than the standard mean (10.66 t ha^{-1}), but the grain yield alone is not sufficient to determine the value of a hybrid, which also depends on the grain moisture at harvest.

Compared with the standards, 12 of the hybrid combinations had lower grain moisture, but this was associated with low grain yield. A further 22 hybrids, which had moisture contents of over 21% combined with lower yields, did not satisfy the criteria raised in the experiment, while the grain moisture contents of 18 combinations did not differ significantly from the mean for the commercial hybrids, but 14 of these had grain yields of below 10 t ha^{-1}, i.e. they yielded significantly less than the standard mean.

All in all, only two hybrids created using DH lines came up to expectations for both traits. These had yields equivalent to those of state registered combinations, while also having similar values of grain moisture content at harvest. It should be noted, however, that these hybrids would not have been granted state registration on the basis of official trials, as the criterion is not to equal the standard mean, but to exceed it.

DH 56 was found to be the best combining line, based on the performance of its hybrids. In previous experiments this line was found to be suitable for the development of regenerated plants, while also passing on its good combining ability (GCA and SCA are traits with good heritability; Jenkins, 1935).

When evaluating the results it must not be forgotten that they were obtained using a very restricted genetic base, involving one inbred line of Iodent origin and genetic materials arising from crossing combinations of an inbred line which has an exotic appearance in Hungary. The 12 DH lines tested only made it possible to carry out an experiment with an extremely low sample number. If the work were expanded to include DH lines with a broader genetic background and a higher number of test crosses, there appears to be every likelihood that maize hybrids of *in vitro* DH origin, capable of out-performing the standard mean, could be developed and granted state registration in the future.

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STUDIES ON SELF-INCOMPATIBILITY IN LOCAL INDIAN CULTIVARS OF RADISH (*Raphanus sativus* L.)

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In the present study on the self-incompatibility in inbred lines of ten local Indian cultivars (Pusa Chetki, Chetki Long, Aushi, Alipur Local White, Jaunpuri, Half Red, Scarlet Red, Chinese Pink, Desi Red and Khasi Kata) of radish (*Raphanus sativus* L.), Pusa Chetki, Chetki Long, Aushi, Alipur Local White and Jaunpuri were classed as self-compatible, Half Red, Scarlet Red and Chinese Pink as intermediate and Desi Red and Khasi Kata as self-incompatible. The highest number of germinated pollen grains and pollen tubes was observed in Pusa Chetki, followed by Alipur Local White, Jaunpuri, Aushi and Chetki Long. The discrepancy in the number of germinated pollen grains in the stigmas may be explained by the inhibitory action of large numbers of self-incompatible pollen grains on the stigma. When two lines, Desi Red and Khasi Kata, were grown under different temperature and photoperiod conditions, no breakdown in self-incompatibility was observed, and the flowering periods of these lines are naturally well synchronized. It is well known that uniform and effective cross-pollination may be of great importance for obtaining a high quantity of hybrid seed in self-incompatible types. To produce single cross hybrid seed, the inbred lines Desi Red and Khasi Kata can be used as parental lines.

Key words: radish, self-incompatibility, hybrid seed production

Introduction

The expression of hybrid vigour in the F_1 generation over the mid-parental value was reported for various morphological and biochemical traits of radish (Singh et al., 1986). The presence of heterosis and the availability of pollination control mechanisms such as self-incompatibility in crop plants have proved to be efficient tools for obtaining higher returns in advanced agricultural economies. The production of hybrid seeds utilizing self-incompatibility is commercially accepted, especially in cauliflower, radish, Brussels sprouts, cabbage and kale (Kalloo, 1988). After evaluation for combining ability, Kononkov et al. (1977) obtained six self-incompatible lines of radish. Hybrids of these lines were 22–65% superior to the initial varieties for root weight and 6–12% for root quality.

Self-incompatibility is the inability of functional pollen to set seed after self-pollination, while in the case of self-compatibility the pollen grains of a flower can pollinate the same flower. The sporophytic system operating in radish consists of multiple allelic series and comprises many S-alleles. S-alleles are effective in preventing self-fertilization. There may be independent action of the alleles or one may be dominant over the others (Kalloo, 1988). The stigmatic surface of a cruciferous flower with a sporophytic self-incompatibility system is covered with a layer of papilla cells and is a so-called 'dry stigma', which inhibits pollen germination and the growth of the pollen tube. To exploit hybrid vigour in crucifers, the identification of self-incompatibility among selected plants and their progenies is of prime importance for breeders (Opena et al., 1988). Information on the nature and extent of self-incompatibility in local Indian radish is very scanty. Therefore, this study was carried out to understand the nature and extent of self-incompatibility in local radish cultivars.

Materials and methods

The experiment was conducted at the Plant Biotechnology Center, Swami Keshwanand Rajasthan Agricultural University, Bikaner, Rajasthan, India, in the 2006–07 and 2007–08 seasons. Bikaner is situated in the hyper-arid, partially irrigated western plain zone of India. It is situated at 28.01° N and 73.22° E at an elevation of 234.70 m above mean sea level. Inbred lines of ten Indian radish cultivars were used in this study. The cultivars were Pusa Chetki, Chetki Long, Aushi, Alipur Local White, Jaunpuri, Half Red, Scarlet Red, Chinese Pink, Desi Red and Khasi Kata. The seeds of these cultivars were collected from farmer's fields and local markets and sown in the field on November 16, 2006 and November 20, 2007.

Seed set analysis

Seed set analysis was used as an estimate for the level of self-incompatibility. Selfing using the hand pollination method was done both in the bud and at anthesis to compare differences in compatibility between these stages. Two flower stalks where blooming started at the bottom were selected for each plant. All the open flowers were removed and the remaining buds were covered with paper bags. Two days after bagging, at least ten open flowers were selfed using fresh pollen from a different flower on the same stalk or from the same flower. For bud selfing, about ten flower buds were selfed using pollen from open flowers on the same stalk. The remaining small buds at the tip were removed.

Since the determination of the level of self-incompatibility is difficult, it is simplified by utilizing the "seed set ratio" (Zuberi and Zuberi, 1981; Kich and Torralba, 1989; Mbinga et al., 1994). The number of seeds obtained from 30 selfings of each type was recorded at harvest and the seed set ratio of the cultivar was determined as follows:

$$\text{Seed set ratio (\%)} = \frac{\text{Seeds from 30 open selfings}}{\text{Seeds from 30 bud selfings}} \times 100$$

The plants were classed as self-compatible if the ratio was 50% or above, intermediate if the ratio was more than 10% but less than 50%, and self-incompatible if the ratio was 10% or less.

Fluorescence microscopy (Wallace, 1979; Opena et al., 1988; Gemmell et al., 1989; Zuberi and Sarker, 1992) was employed to test the self-incompatibility mechanism by observing pollen germination on the stigma and pollen tube growth in the style. Three types of pollination, bud selfing, open selfing and cross-pollination, were performed on a total of ten flowers per treatment. Cross-pollinations were done using pollen from the cultivar Taski, a cross involving Tangil Local

as male parent. The pollinated flowers were removed from the plants 24 hours after pollination. The pistils were fixed in acetic acid and alcohol (1:3 v/v) for 24 hours and then transferred to 70% ethanol. They were then placed in 1 N NaOH at 60°C for about 25 min to soften the tissues, and then stained overnight with 0.1% aniline blue containing tri-potassium orthophosphate, following the method described by Kho and Baer (1968). The samples were smeared on glass slides and observed under a fluorescence microscope.

The number of germinated pollen grains per stigma and the number of pollen tubes near the base of the style were recorded. Pollinations were scored as compatible where pollen germination was abundant and more than 16 pollen tubes were observed to enter the stigmatic papilla after 24 hours of pollination, as incompatible where less than 4 pollen tubes were seen entering the papilla, and as intermediate where 4 to 16 pollen tubes were observed to enter the stigma (Zuberi and Sarker, 1992).

Results and discussion

Seed set analysis

The results of seed set analysis are shown in Table 1. Based on the mean percentage seed set ratio, the cultivars Pusa Chetki, Chetki Long, Aushi, Alipur Local White and Jaunpuri were classed as self-compatible, Half Red, Scarlet Red and Chinese Pink as intermediate (having a 10–50% seed set ratio) and the remaining two cultivars, Desi Red and Khasi Kata, as self-incompatible (< 10% seed set ratio).

Nieuwhof (1985) also reported that the incompatibility mechanism present in European Early Red and related early radish cultivars was rather weak, as most of the plants gave good seed yield even after four generations of selfing. The results reported here indicate that out of 10 local radish cultivars five were self-compatible, three intermediate and two self-incompatible. In *Brassica napus*, Gemmell et al. (1989) identified self-incompatible lines by seed set analysis and pollen tube counts on intra- and inter-line pollinations. They classified the lines as self-incompatible (seed set/flower from 0 to 0.40) to fully cross-compatible (seed set/flower between 10 and 20). None of the cultivars in the present study was found to be fully self-incompatible, indicating that the self-incompatibility mechanism in the local radish cultivars tested here is rather weak.

The traditional method of detecting self-incompatibility is through seed set analysis. However, seed set analysis takes more than a month from pollination to harvest, inadvertent cross-fertilization and seed mixing at harvest are hard to eliminate completely, and environmental factors may further influence the self-incompatibility reaction. Therefore, several workers developed possible alternatives to seed set analysis. Sampson (1964; 1967) developed a technique for counting empty pollen grains on the radish stigma using a fluorescence microscope to readily display the pollen tubes that had penetrated the style, providing a direct and immediate measure of incompatibility (Opena et al., 1988).

Table 1
Estimates of self-incompatibility in radish cultivars through seed set analysis in two years

Name of cultivars	No. of seeds in open selfing*		No. of seeds in bud selfing*		Percentage fertility		Mean percentage fertility	Prediction
	2006–07	2007–08	2006–07	2007–08	2006–07	2007–08		
Pusa Chetki	107	117	154	127	69	92	81	Self-compatible
Chetki Long	90	87	119	116	76	75	76	Self-compatible
Aushi	40	69	64	105	63	66	65	Self-compatible
Alipur Local White	55	46	103	92	53	50	52	Self-compatible
Jaunpuri	52	36	87	93	60	39	50	Self-compatible
Half Red	49	37	125	89	39	42	41	Intermediate
Scarlet Red	10	11	39	39	26	28	27	Intermediate
Chinese Pink	63	33	163	129	39	26	33	Intermediate
Desi Red	6	6	89	69	7	9	8	Self-incompatible
Khasi Kata	4	3	94	62	4	5	5	Self-incompatible
Mean	47.6	44.5	103.7	92.1	46.0	48.3		

* Total of 30 pollinations

Pollen germination and pollen tube growth

The results of pollen germination and pollen tube growth are shown in Table 2. The data revealed that both the cultivar and type of pollination had a substantial effect on pollen germination and pollen tube growth in both years.

The highest number of germinated pollen grains and pollen tubes was observed in Pusa Chetki followed by Alipur Local White, Jaunpuri, Aushi and Chetki Long. The lowest number of germinated pollen grains per stigma and pollen tubes per style were found in the cultivar Khasi Kata in both years. Based on the mean number of pollen tubes per style, the cultivars Pusa Chetki, Alipur Local White, Jaunpuri, Aushi and Chetki Long were grouped as self-compatible, Half Red, Scarlet Red and Chinese Pink as intermediate and Desi Red and Khasi Kata as self-incompatible. Thus, these results are more or less in accordance with those obtained by seed set analysis.

It is clear from Table 2 that the type of pollination had a noticeable influence on the germination of pollen grains per stigma and the number of pollen tubes entering the style in both seasons. The highest number of pollen tubes per style was due to cross-pollination in both years, but was not much different to that recorded in bud selfing. Open selfing yielded the lowest number of germinated pollen grains per stigma and pollen tubes per style. In the present study, the discrepancy in the number of germinated pollen grains in the stigmas may be explained by the inhibitory action of large numbers of self-incompatible pollen grains on the stigma.

The stability of self-incompatibility in the two cultivars Desi Red and Khasi Kata, the plants of which showed a fertility ratio of 0.0 to 8.7, might be controlled by a major gene. Apart from having a less than 10% self-fertility ratio, it is important for an inbred parent line to have the ability to produce enough seeds via bud pollination to maintain it. This criterion was met by the two inbred lines.

Table 2
Estimates of self-incompatibility in radish cultivars in two years by observing pollen germination on the stigma and pollen tube growth in the style using fluorescence microscopy

Treatment	No. of germinated pollen grains per stigma*		No. of pollen tubes per style*			Judgement
	2006–07	2007–08	2006–07	2007–08	Mean	
Pusa Chetki	55.3	42.3	22.3	23.6	22.9	Compatible
Chetki Long	44.8	29.9	19.6	18.2	19.0	Compatible
Aushi	31.5	33.6	21.8	19.6	21.0	Compatible
Alipur Local White	35.2	22.6	23.3	20.9	22.0	Compatible
Jaunpuri	33.9	29.3	20.6	21.8	21.2	Compatible
Half Red	17.6	12.4	14.2	13.5	13.8	Intermediate
Scarlet Red	17.3	21.2	10.8	16.9	13.8	Intermediate
Chinese Pink	18.9	19.1	10.6	15.8	13.2	Intermediate
Desi Red	11.8	13.0	9.3	8.4	8.8	Incompatible
Khasi Kata	32.0	28.0	6.2	4.0	5.1	Incompatible
Type of pollination						
Bud selfing	36.4	32.3	19.9	18.3	19.1	–
Open selfing	21.2	19.8	6.4	5.8	6.1	–
Cross-pollination	39.9	34.5	23.2	21.8	22.5	–

*Mean of 10 flowers

Hybrid seed production using self-incompatibility involves the development and maintenance of parents which are homozygously self-incompatible but cross-compatible, so the degree of self-incompatibility and stability in the inbred lines is of utmost importance. This is largely dependent on environmental factors, especially temperature. When Desi Red and Khasi Kata were grown under different temperature and photoperiod conditions, no breakdown in self-incompatibility was observed. The flowering periods of Desi Red and Khasi Kata are naturally well synchronized. It is well known that uniform, effective cross-pollination may be of great importance for obtaining a high quantity of hybrid seed in self-incompatible types. The two inbred lines, Desi Red and Khasi Kata, can be used as parental lines to produce single cross hybrid seed. The lines should be grown in alternate rows or with two to four rows of each, and adequate insect pollination should be ensured. The F_1 populations of such hybrids will be uniform.

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EFFECT OF PRE-UTILISATION AND HARVEST TIME ON THE QUANTITY AND QUALITY OF FODDER ON EXTENSIVE PASTURES

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No significant studies have yet been reported in Central Europe on the yield and quality of winter harvest pastures. The aim of the research was to collect information about the effect of pre-utilisation (June, July and August) and winter harvest date (November, December, January) on the quantity and quality of fodder from *Festuca arundinacea* stands. The dry matter, energy, ADF, ADL and ergosterol contents of the yield were examined and it was found that:

1. A shorter regeneration period between harvests resulted in lower dry matter levels, but also in a higher energy concentration and lower ergosterol concentration.
2. The yield and energy concentrations decreased, whereas the ADF, ADL and ergosterol concentrations increased as the winter progressed.
3. Fodder harvested in November produced the best results in terms of yield quantity and quality.

The highest yield and energy values were thus achieved by harvesting in November, regardless of pre-utilisation. Despite the cold and the long period of snow cover, the energy values of samples harvested in December and January showed no significant decrease. The weather conditions were more important for fodder quantity and quality than the frequency or date of harvesting. Thus, under the continental climatic conditions in Hungary, extensive utilisation, until late November or early December, is recommended.

Key words: winter harvest, pre-utilisation, DM yield, energy concentration, ergosterol

Introduction

One form of extensive grassland use is year-round grazing by dairy cows or beef cattle herds. This form of utilisation has become more important recently, particularly for peripheral sites in Central Europe (Opitz von Boberfeld, 2001). The advantage of the method is its low cost (Deblitz et al., 1993; Bauer, 1996). Costs related to stall housing are eliminated; in addition, labour requirements are diminished (van Keuren, 1970).

Another positive aspect of the method is that it enables farmers to produce fodder in an environmentally friendly and energy-efficient way, resulting in high quality, healthy end-products (Langholz, 1992). The most important costs are represented by storing and transport in the winter. These costs may be reduced, however, if the grassland remains in use not only in the vegetation period, but also during the winter months.

Experience on winter grazing has been collected for decades in the Atlantic climate area. In Hungary, many studies have been published on the effect of fertilization on grasslands (Kádár et al., 2007; Füleky, 2008; Kádár, 2008), but the results apply only to the vegetation period. Nagy et al. (2006), Czeglédi and Radácsi (2005) and Pajor et al. (2007) observed the role of pastures in grazing by various animal species, but the authors did not examine the winter months. Under continental conditions in North America, winter grazing has a key role (Allen et al., 1989; Bartholomew et al., 1997). The yield of fodder grasses depends not only on the characteristics of the species and competition, but also on weather conditions and the type and frequency of use (Voigtländer, 1987). Experiments conducted in Germany and Great Britain show that winter fodder production greatly depends on the timing of the last summer harvest in the vegetation period and the date of winter harvest (Gerrish et al., 1994; Opitz von Boberfeld and Wolf, 2002). According to Wolf (2002), the frequency of harvesting in the vegetation period does not play a central role. According to some authors, if the last summer harvest is delayed, the yield of *Festuca arundinacea* will decrease (Gerrish et al., 1994; Opitz von Boberfeld and Wolf, 2002). With the progress of winter, fodder values also decrease (Bartholomew et al., 1997). The main features of the harvested growth are energy concentration and digestibility, these being the most important performance indicators of a given production site (Opitz von Boberfeld, 1994). According to Lindgren and Lindberg (1988), if the harvest is delayed, the daily decrease in the energy concentration values will be 0.1 MJ metabolic energy. Thus, extensive grassland use combined with delayed first harvest and low harvest frequency may impair fodder quality (Dahmen and Kühbauch 1990; Common et al., 1991; Käding et al., 1993; Opitz von Boberfeld, 1996). With elongated growing periods and rough winter weather, digestibility decreases (Collins and Balasko, 1981); the later the fodder is harvested in the winter, the less digestible it will be (Collins and Balasko, 1981; Hitz and Russell, 1998). In investigations made by Archer and Decker (1977) in the autumn, a negative correlation was found between NDF, ADF and lignin contents and digestibility in *Festuca arundinacea* stands. In winter experiments, significant negative correlations were found between the concentration of water-soluble carbohydrates and digestibility in *Festuca arundinacea* stands (Collins and Balasko, 1981). In the experiments of Opitz von Boberfeld (1994), plant aging caused a daily increase of about 0.2% in the ADF and crude fibre concentration.

The aim of the present research was to collect information about the effect of pre-utilisation (June, July, and August) and winter harvest date (November, December, January) on the quantity and quality of fodder.

Materials and methods

The experiments were conducted in the Research Centre of the Institute of Crop Production, Szent István University, Gödöllő (Hungary), 30 km east of Budapest and 207 m above sea level, on brown forest soil. The plots were fertilized with 50 kg ha^{-1} N after pre-utilisation. Weather data are shown in Table 1.

The area was covered by snow for 8 days during the first winter and 36 days during the second.

The main plant species was *Festuca arundinacea* (90%). Plots measuring 2×3 m were laid out in a Latin square in three replications in June 2000 (Table 2).

The plots were harvested leaving a 5 cm stubble and samples were taken from the yield of each plot for analytical purposes. To determine fodder quality, the energy concentration was expressed as the net energy of lactation (NEL), which was estimated on the basis of gas production (Steingass and Menke, 1986) and the concentration of crude protein (Anonymous, 1997) and crude fat (Anonymous, 1997). The concentrations of neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (ADL) (Anonymous, 1997) were also determined. The amount of water-soluble carbohydrates was determined using the anthrone method (Yemm and Willis, 1954), while the ergosterol concentration was established using high performance liquid chromatography (HPLC) (Schwadorf and Müller, 1989; Anonymous, 1993). The data were analysed using ANOVA and Fisher's LSD at $P < 0.05$.

Table 1
Weather data for the experimental station in Gödöllő (207 m above sea level)

	VI	VII	VIII	IX	X	XI	XII	I	II	III	IV	V
1st year °C	21	20	22.3	15	13	7.6	1.6	–0.2	0	6.9	10	17
1st year mm	6	106	8	23	6	64	40	95	31	74	28	24
2nd year °C	17	21.1	22.5	14	13	1.8	–5.1	–0.1	4	6.9	11	18
2nd year mm	67	134	63	95	4	40	26	10	15	16	30	81

Table 2
Variants in the experiment

Factors	Stages
1. Winter harvest	1.1 November
	1.2 December
	1.3 January
2. Summer harvest	2.1 June
	2.2 July
	2.3 August
3. Year	3.1 2000/2001
	3.2 2001/2002

Results

The dry matter yields showed a significant difference in the two years (Fig. 1).

The amounts harvested in November in both 2001 and 2002 were twice those harvested at any other date. In the first winter, harvesting in November following pre-utilisation in June and July produced a significantly higher yield. In the second winter, harvesting in November produced a significantly higher yield when it was preceded by pre-utilization, regardless of the date of pre-utilization.

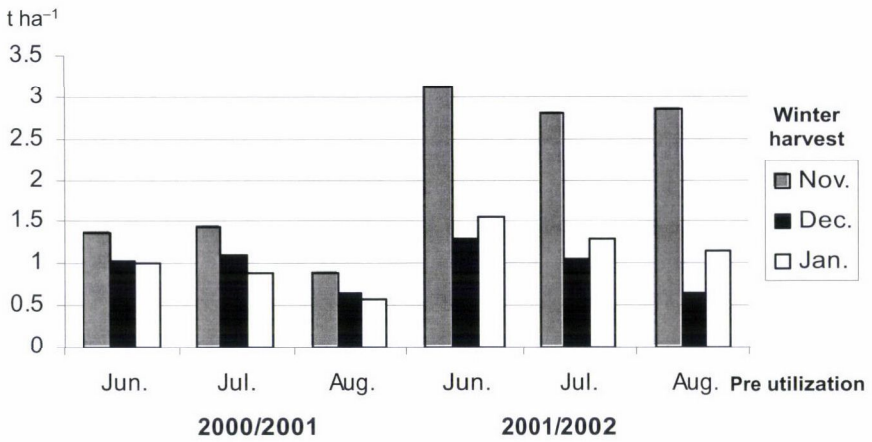

Fig. 1. Relationship between dry matter yields and the dates of pre-utilisation and winter harvest ($LSD_{5\%}$ 2000/2001: 0.41; 2001/2002: 0.62)

The net energy of lactation (NEL) values of the fodder at various harvesting and pre-utilisation dates (Fig. 2) indicated that harvesting time had the greatest influence on NEL. The highest NEL values were measured in November in both years.

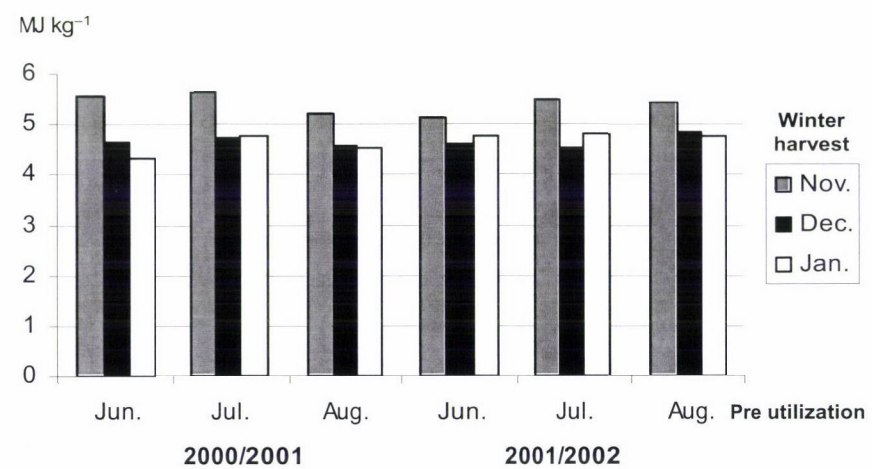

Fig. 2. Dependence of net energy of lactation on pre-utilisation and winter harvest dates ($LSD_{5\%}$ 2000/2001: 0.69; 2001/2002: 0.55)

The harvesting date had a clear effect on the NDF concentration in both years (Table 3), the lowest concentrations being found in November, while there was no significant difference between the values recorded in December and January. Pre-utilisation had only a minor effect on the NDF concentration. The lowest ADF concentrations were found in November in both years, so the significant effect of the harvesting date was again obvious. Significant differences were found between the earliest pre-utilisation date (June) and later dates (July, August). The ADL concentration was the lowest in November and highest in January in both years (Table 3), confirming the significant effect of harvesting date. The interaction between pre-utilisation and harvesting date was significant in both years. If the pasture was first harvested in June or July the ADL concentrations increased with each later harvesting date, whereas after August pre-utilisation the yield of the December harvest had the highest ADL concentration.

The statistical analysis showed that the harvesting time had the strongest effect on the concentration of water-soluble carbohydrates ($p<0.05$). The highest values were recorded in November and the lowest in January in the first year, but in the second year no significant difference could be detected between the values measured in December and January.

The ergosterol concentrations were nearly twice as high in the first year as in the second year, increasing with each harvest date. The concentrations measured in November were low in the case of June and July pre-utilisation. The values increased again in December and remained at the same level in January. In samples taken after August pre-utilisation, the ergosterol levels increased until December, but the January data showed a significant decrease in the concentration.

Table 3

Nutrient contents (%) as a function of year, pre-utilization and winter harvest date

Pre-utilisation		June			July			August			$LSD_{5\%}$
Winter harvest		Nov.	Dec.	Jan.	Nov.	Dec.	Jan.	Nov.	Dec.	Jan.	
NDF	2000–2001	59.4	66.5	72.5	60.2	67.2	69.8	60.7	66.9	68.4	4.20
(as % of DM)	2001–2002	63.2	74.4	72.2	61.3	73.1	70.8	58.9	72.6	70.1	3.20
ADF	2000–2001	33.8	35.9	40.7	33.9	37.2	37.6	33.4	39.2	36.5	3.95
(as % of DM)	2001–2002	37.1	41.7	43.9	33.5	42.0	40.5	32.5	41.7	39.6	2.27
ADL	2000–2001	4.9	5.3	8.1	4.6	5.2	6.2	4.8	6.8	5.8	1.55
(as % of DM)	2001–2002	6.4	5.8	10.7	4.7	6.4	6.5	4.4	6.7	5.7	2.03
Water-soluble	2000–2001	13.0	7.5	4.3	14.5	8.2	4.8	13.6	7.6	5.5	1.49
carbohydrates	2001–2002	11.5	4.5	5.9	12.3	4.7	5.3	13.2	4.6	5.6	2.05
Ergosterol	2000–2001	108.0	172.3	234.3	102.3	134.0	201.0	144.3	186.7	215.0	41.14
(mg kg DM^{-1})	2001–2002	51.3	98.0	102.0	34.0	104.7	83.0	27.3	131.0	63.3	25.58

Discussion

The yield differed significantly in the two years. In 2000/2001 the winter harvest yielded about half as much as in the following year. Harvesting in November resulted in significantly higher yields. In 2001/2002, the yield harvested in November was more than twice as much as in the first year. This could be explained by the favourable weather conditions (Table 1), as the amount of precipitation in September (95 mm) resulted in enhanced grass growth. November utilisation produced a significantly higher yield compared with December and January. The significant effect of the timing of winter harvests could again be explained by the weather conditions. Under continental climatic conditions, the grass stops growing from the middle of November, as the average temperature falls to around zero and a considerable loss of green mass is also to be expected. It can thus be concluded that under continental conditions the weather has a much more pronounced effect on yield quantity than the date and frequency of harvesting.

With the progress of winter, energy contents and digestibility decreased, as also observed by Collins and Balasko (1981), Hitz and Russell (1998) and Opitz von Boberfeld and Wolf (2002). Opitz von Boberfeld (1994; 1996) attributed this to an age-related increase in the ratio of sclerenchyma in the plants. Collins and Balasko (1981) and Opitz von Boberfeld and Wolf (2002) found better digestibility indicators in younger stands. In the present work, the lowest ADF and ADL concentrations were found in November in both years. The fact that the highest concentrations were not measured in January could be explained by the cultivation of a winter sward, which may have a positive effect on the yield quality in a mild winter. Due to the heterogeneous chemical composition, no strict quality limits have been established for the digestibility of the sclerenchyma (Wilman et al., 1996). The quantities of lignin and SiO_2 in the cell walls may also influence digestibility (van Soest and Jones, 1968). The same correlation between ADF concentrations and digestibility was found in both years (Table 4). A similar correlation was found by Archer and Decker (1977), who reported a close correlation between ADF concentrations and OS digestibility in autumn and late autumn measurements. There was a linear correlation ($r > 0.6$) between the ADL concentration and the energy content (Table 4), which can be explained by the decrease in digestibility caused by lignin. The aging of the sward influences both factors. At the beginning of winter, the amount of water-soluble carbohydrates usually drops from a maximum to a minimum level, where it remains until the next spring (Powell et al., 1967). In addition, the ratio of dead tissues increases, the digestibility of which is much lower than that of live plant tissues (Archer and Decker, 1977). With the progress of winter, vitality decreases and the rate of aging increases. The December harvest in the second year was an exception; the continuous snow cover for 31 days increased the ergosterol concentration (Table 3). According to

Wolf (2002), in the case of older grass (i.e. after early pre-utilisation) the ergosterol levels are higher. In the present experiment, young growth (August pre-utilisation) had an unusually high ergosterol concentration in the wet, rainy months of November 2000 and December 2001. These results show that higher amounts of precipitation create a more favourable habitat for fungi in the young sward. The close correlation observed between the ADF and ADL concentrations and the ergosterol concentration (Table 4) can be explained by the increased utilisation of easily digestible plant material by fungi. This was also confirmed by the increase in energy values with the progress of winter and by the fact that mycosis occurred mostly in older vegetation. Opitz von Boberfeld (1996) also found that ergosterol concentrations increased with age.

In summary, it can be concluded that on areas with a continental climate like that in Gödöllő, the grazing period can be extended to the end of November on *Festuca arundinacea* swards. From December onwards, forage indicators will be mostly influenced by the weather, so more care is required when planning for this period.

Table 4
Linear correlation (r) between indicators of forage quality, n = 27

		ADL	Water-soluble carbohydrates (WSC)	Ergosterol	Net energy of lactation (NEL)
ADF	2000/2000	+0.81**	–0.71**	+0.64**	–0.73**
	2001/2002	+0.70**	–0.93**	+0.84**	–0.85**
	ADL	2000/2001	–0.61**	+0.71**	–0.63**
		2001/2002	–0.53**	+0.51**	–0.69**
			WSC 2000/2001	–0.84**	+0.86**
			2001/2002	–0.86**	+0.84**
				Ergosterol 2000/2001	–0.78**
				2001/2002	–0.70**

**Significant at the 0.01 level of probability

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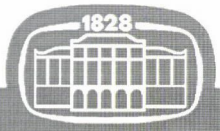

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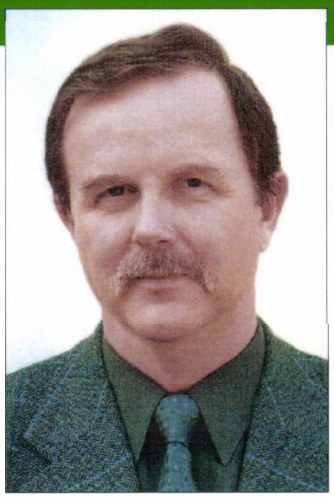

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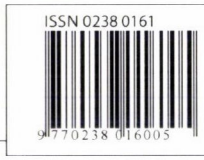

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CONTENTS

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VISUALIZATION OF U AND M GENOME CHROMOSOMES BY MULTICOLOUR GENOMIC *IN SITU* HYBRIDIZATION IN *Aegilops biuncialis* AND *Triticum aestivum–Ae. biuncialis* AMPHIPLOIDS

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The multicolour genomic *in situ* hybridization (mcGISH) method was improved in order to visualize the U^b and M^b genomes of *Aegilops biuncialis* Vis. (2n=4x=28, $U^bU^bM^bM^b$). Hybridization probes prepared from the diploid U and M genome donors, *Ae. umbellulata* and *Ae. comosa*, resulted in clear hybridization signals on the U and M chromosomes in *Ae. biuncialis*. The random primed labelling method made it possible to decrease the blocking ratio to 1:30. McGISH allowed the simultaneous discrimination of individual *Ae. biuncialis* genomes and wheat chromosomes in γ-irradiated *Triticum aestivum–Ae. biuncialis* amphiploids (2n=70; $AABBDDU^bU^bM^bM^b$). Dicentric chromosomes, terminal and interstitial translocations and centric fusions were detected in the irradiated generation. The irradiation-induced wheat–*Ae. biuncialis* intergenomic translocations will facilitate the successful introgression of useful agronomic traits into bread wheat.

Key words: *Triticum aestivum*, *Aegilops biuncialis*, amphiploid, mcGISH, γ-irradiation, intergenomic translocations

Introduction

Aegilops biuncialis Vis. (2n=4x=28; $U^bU^bM^bM^b$) is an allotetraploid species having good tolerance against biotic (Damania and Pecetti, 1990; Makkouk et al., 1994) and abiotic stresses such as cold, salt and heat stresses (Ekmekci and Terzioglu, 2002; Dulai et al., 2005; Colmer et al., 2006). Accessions originating from semi-desert habitats can also be used as gene sources to improve the drought tolerance of wheat (*Triticum aestivum* L.) (Molnár et al., 2004).

The first stable stage in the chromosome-mediated transfer of favourable genes from *Ae. biuncialis* into wheat is the production of wheat–*Ae. biuncialis* amphiploids (2n=10x=70; $AABBDDU^bU^bM^bM^b$) followed by the production of addition and translocation lines. This gene transfer process requires the

production of many backcrossed generations in order to decrease the *Aegilops* chromosome number. Therefore, it is essential to know the number of wheat and *Aegilops* chromosomes and the genomic origin of each in the subsequent generations. It is also desirable to use a suitable method to detect wheat–*Aegilops* intergenomic translocations involving as little alien chromatin as possible.

Since the first successful gene transfer from *Aegilops umbellulata* Zhuk. to wheat (Sears, 1956), ionising irradiation (such as X- and γ-rays) has been widely applied to crop species for the production of interspecific translocations (Knott, 1987; Friebe et al., 1991; Forsberg et al., 1998; Riera-Lizarazu et al., 2000). Irradiation methods have several practical advantages over recombination-mediated strategies: (1) theoretically, any alien chromosome segment could become inserted into a wheat chromosome without losing any wheat chromatin, and (2) the efficiency with which interspecific translocations are formed is not affected by the pairing potential between alien and wheat chromosomes (Jiang et al., 1994). The irradiation of wheat–*Ae. biuncialis* amphiploids may thus yield a large number of intergenomic translocations between the *Aegilops* and wheat genomes.

Multicolour genomic *in situ* hybridization (mcGISH) using differentially labelled total genomic DNA probes enables the parental genomes to be discriminated in allopolyploid plants (Mukai et al., 1993; Belyayev et al., 2001) and intergenomic chromosome rearrangements to be detected. The simultaneous visualization of individual wheat genomes and alien chromatin in interspecific hybrids and derivatives has also been reported (Sánchez-Morán et al., 1999; Han et al., 2003). Benavente et al. (2001) individually distinguished the U^o and M^o genomes of *Aegilops ovata* L. in durum wheat–*Ae. ovata* amphiploids using the total genomic DNA of *Ae. umbellulata* and *Ae. comosa* Sm. in Sibth. & Sm. as U and M genomic probes. However, no information is available on the parallel discrimination of the U^b and M^b genomes of *Ae. biuncialis* from bread wheat chromosomes.

The present study reports the simultaneous discrimination of the U^b and M^b genome chromosomes in *Ae. biuncialis* and in the wheat genetic background by mcGISH. This technique was used to investigate the formation of intergenomic chromosome rearrangements in γ-irradiated wheat–*Ae. biuncialis* amphiploids.

Materials and methods

Plant material

The *Aegilops biuncialis* Vis. accession MvGB642 was used to improve the multicolour genomic *in situ* hybridization (mcGISH) method. *Triticum aestivum–Ae. biuncialis* amphiploids were produced by crossing the winter wheat genotype Mv9kr1 (Molnár-Láng et al., 1996) with the *Aegilops biuncialis* accession ICAG400808, kindly provided by the ICARDA Gene Bank, Syria. The F_1 hybrids were treated with colchicine and the seeds of the third selfed generation were used

for the present study. Dry seeds of the amphiploid were irradiated with ^{60}Co γ-rays at a dosage of 100 Gy. Non-irradiated seeds were used as the control. The irradiated and control seeds were germinated, and were analysed by mcGISH to identify intergenomic rearrangements involving wheat and the U^b and M^b genome chromosomes of *Ae. biuncialis*.

Cytological preparation, DNA probes and labelling

Chromosome preparations were made from *Ae. biuncialis* and from wheat–*Ae. biuncialis* amphiploid root tips according to the method described by Jiang et al. (1994). Total genomic DNA from *Ae. umbellulata* (UU) and *Ae. comosa* (MM), the diploid progenitors of *Ae. biuncialis*, were labelled with biotin (biotin-16-dUTP, Roche) and digoxigenin (digoxigenin-11-dUTP, Roche) by random priming and used as U^b and M^b genome probes, respectively. Unlabelled genomic DNA from durum wheat (*Triticum turgidum* ssp. *durum* L., 2n=4x=28; AABB) was sheared by autoclaving and used as a block. Digoxigenin and biotin were detected using anti-digoxigenin-rhodamine Fab fragments (Roche) and streptavidin-FITC (Roche), respectively.

McGISH procedure

Pretreatments and stringency washings of the slides were carried out as described by Schneider et al. (2005). The hybridization mixture (25 μL per slide), including 70 ng of each U^b and M^b genome probe and 2.1 μg competitor DNA, the denaturation and hybridization conditions and the detection of hybridization signals were described previously by Sepsi et al. (2008). Images were acquired through a Zeiss Axioskop-2 fluorescence microscope using a Plan Neofluar oil objective ×63, N.A. 1.25 (Zeiss, Oberkochen, Germany) equipped with filter sets appropriate for DAPI (Zeiss filter set 02), FITC and Rhodamin (Zeiss filter set 24) with a Spot CCD camera (Diagnostic Instruments, Sterling Heights, Michigan, USA). The images were compiled with Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

Results

Visualization of U^b and M^b chromosomes in Ae. biuncialis

The rapid method of genomic *in situ* hybridization with high hybridization temperature (65°C) was modified in order to visualize the U^b and M^b chromosomes of *Ae. biuncialis*. The critical steps in the optimization procedure were the modification of the hybridization conditions to 16 h at 42°C, the use of differentially labelled genomic DNA from the diploid progenitors, *Ae. comosa* and *Ae. umbellulata*, as probes instead of *Ae. biuncialis*, and indirect probe labelling with digoxigenin and biotin by random priming. Tests were made on the effect of the blocking ratio on the discrimination of U^b and M^b genomes and the best hybridization signal was obtained with a blocking ratio of 1:30. As a result of GISH optimization it was possible to visualize the U^b and M^b genomes in the same root tip meristem cell of *Ae. biuncialis* (Fig. 1A).

*McGISH analysis of the irradiated wheat–*Ae. biuncialis *amphiploids*

Untreated and γ-irradiated wheat–*Ae. biuncialis* amphiploids were also analysed with mcGISH. Using differentially labelled total genomic DNA from *Ae. umbellulata* and *Ae. comosa*, the U^b- and M^b-genome chromosomes were clearly discriminated as showing red and green fluorescence, respectively, while the unlabelled A-, B- and D-genome chromosomes of wheat were brown (Fig.

1B, C, D). Based on the colour discrimination it was possible to detect intergenomic chromosome rearrangements between wheat and U^b (Fig. 1C; D1, 4, 7), wheat and M^b (Fig. 1B; D2, 3, 5, 6, 8) and U^b and M^b chromosomes (Fig. 1C). The following chromosomal aberrations were found: dicentric chromosomes (Fig. 1B; C; D1, 2), terminal translocations (Fig. 1C; D3, 4), interstitial translocations (Fig. 1D5, 6) and centric fusions (Fig. 1D7, 8).

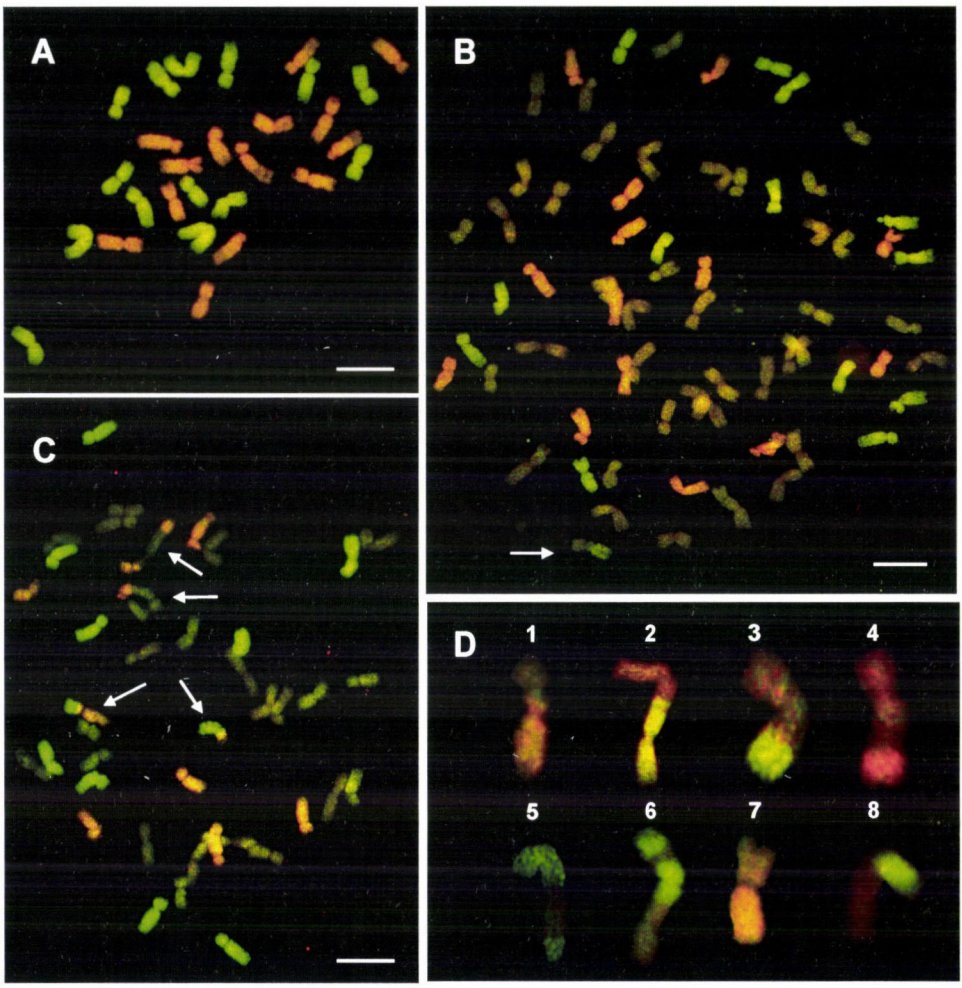

Fig. 1. Multicolour GISH discrimination of U^b-genome (orange) and M^b-genome (green) chromosomes in a root-tip cell at mitotic metaphase in *Ae. biuncialis* (A) and in irradiated wheat–*Aegilops biuncialis* amphiploid plants (B, C). In the irradiated amphiploids the intergenomic translocations are indicated by arrows. The irradiation-induced chromosome aberrations involving U^b-genome chromosomes (D1, 4, 7), M^b-genome chromosomes (D2, 3, 5, 6, 8) and wheat chromosomes are summarised in Fig. 1D. Dicentric chromosomes (D1, 2), terminal translocations (D 3, 4), interstitial translocations (D5, 6) and centric fusions (D7, 8) are detected. Scale bar = 10 μm.

Discussion

The aim of the study was to improve the genomic *in situ* hybridization method (GISH) in order to visualize *Ae. biuncialis* chromosomes in a wheat genetic background. Although the U^g and M^g genome chromosomes of *Ae. geniculata* were previously identified by GISH (Benavente et al., 2001; Aghaee-Sarbarzeh et al., 2002) in another study, it proved difficult to distinguish the U and M chromosomes from the A, B and D chromosomes of wheat when *Ae. biuncialis* genomic DNA was used as a probe (Molnár et al., 2005). On the other hand, the application of probes prepared from the diploid U and M genome donors, *Ae. umbellulata* and *Ae. comosa*, resulted in clear, unambiguous hybridization signals on the U and M chromosomes in *Ae. biuncialis* and in wheat–*Ae. biuncialis* amphiploids. The use of U and M genomic probes differentially labelled by the random primed method made it possible to decrease the blocking ratio to 1:30. In a previous study, when nick-translated U or M genomic probes were used, the reproducibility of the results was poor at blocking : probe DNA ratios lower than 200:1 (Molnár et al., 2005).

The present results confirm that mcGISH can be used to discriminate the U and M genome chromosomes of tetraploid *Aegilops* species in a wheat background (Benavente et al., 2001) and to detect chromosome structural changes induced by irradiation in wheat–*Ae. biuncialis* amphiploids. In the irradiated amphiploids, dicentric chromosomes, terminal translocations, interstitial translocations and centric fusions were detected, in agreement with observations on peripheral blood lymphocytes exposed to ionizing irradiation (Beskid et al., 2006). Friebe et al. (1996) and Badaeva et al. (2007) reviewed several wheat–alien and wheat–wheat translocations including terminal and interstitial translocations. The formation of dicentrics as a result of irradiation is a well-documented phenomenon in animals and humans (Natarajan, 2002). The formation of centric fusions in irradiated *T. aestivum–Ae. biuncialis* amphiploids suggests that the centromeric region of wheat and *Aegilops* chromosomes is sensitive to irradiation-induced breakage. A similar sensitivity of the centromeric region to breakage was reported in *Sordaria* (Leblon et al., 1986), tomato (Gill, 1983), pearl millet (Rao and Koduru, 1977), barley (Kunzel et al., 2001) and wheat (Badaeva et al., 2007). The frequent occurrence of translocations involving the centromeric regions of *Sordaria macrospora* was attributed to the AT-rich repetitive DNA sequences (Blackburn and Szostak, 1984). According to these authors, double-strand breaks and crossing over between small homologous sequences during the repair of the DNA lesions could result in translocations. On the basis of its C-banding pattern, the U genome of *Ae. umbellulata* is one of the most heterochromatic within the *Aegilops* genus, while the M genome of *Ae. comosa* has a medium C-heterochromatin content (Badaeva et al., 1996). It can thus be hypothesized that

the heterochromatin content of the centromeric and near-centromeric regions may account for the formation of U^b–wheat rather than M^b–wheat centric fusions.

The study presented here confirms previous findings suggesting that the introgression of alien chromosome segments into wheat from closely related species is very efficient when sexual crossing is followed by irradiation-induced mutagenesis (Sears, 1956). The interspecific translocations produced here are expected to be stabilized in later BC progenies as a set of introgression lines carrying few but distinct rearrangements, which it is planned to characterize using cyto-molecular tools. The wheat–*Ae. biuncialis* amphiploids used for this study exhibited good drought tolerance (Molnár et al., 2008). The genetic stocks developed from irradiated amphiploids thus represent very useful intermediate materials to facilitate successful introgression in wheat breeding programmes.

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INDUCTION OF WHEAT/BARLEY TRANSLOCATIONS BY IRRADIATION AND THEIR DETECTION USING FLUORESCENCE *IN SITU* HYBRIDIZATION

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The aim of the present study was to test the efficiency of gamma irradiation in inducing translocations between wheat and barley genomes using addition lines. The Martonvásári 9 kr1–Igri disomic addition set, previously produced in Martonvásár, was irradiated with gamma rays. The pattern of irradiation-induced intergenomic chromosome rearrangements was analysed in the mutagenized (M_0) generation by genomic *in situ* hybridization (GISH). Centric fusions and a wide variety of reciprocal, terminal and interstitial translocations were frequently induced. The intergeneric translocations produced here are expected to be stabilized in later backcross progenies as a set of introgression lines carrying few but distinct rearrangements.

Key words: *Triticum aestivum, Hordeum vulgare,* winter wheat–winter barley addition lines, *in situ* hybridization, genetic stability, gamma irradiation, translocations

Introduction

Bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) are two of the most important cereal crops in the world. Due to its desirable traits (e.g. tolerance to drought and soil salinity, special nutritional quality parameters and earliness), barley is a promising source for increasing the genetic diversity of common wheat. The prerequisite for transferring agronomically important characteristics from alien species to wheat via conventional cytogenetic procedures is the production of translocation lines from additon or substitution lines. Recombinants with host plant chromosomes may occasionally be produced from addition lines through spontaneous translocations. For instance, a high rate of such translocations was observed in the progenies of seven monosomic wheat–rye addition lines (Ren et al., 1990). In most cases translocations are the result of chromosome manipulation using cytogenetic methods or irradiation with ionizing radiation.

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Recombination between specific barley chromosomes originating from Chinese Spring–Betzes addition lines (Islam et al., 1981) and their wheat homoeologues was induced successfully for the first time by Islam and Shepherd (1992) using the Chinese Spring *ph1b* mutant (Sears, 1977). Six recombinants involving the 6HL and six involving the 3HL chromosome arms were isolated using this method. Sherman et al. (2001) also used the Chinese Spring–Betzes addition set to utilize the effect of the mutant *ph* gene to produce recombinants involving barley chromosomes 4H and 5H.

Since the first successful gene transfer from *Aegilops umbellulata* Zhuk. to wheat (Sears, 1956), ionizing irradiation (such as X- and gamma-rays) has been widely applied to crop species for the production of interspecific translocations (Knott, 1987; Friebe et al., 1991; Forsberg et al., 1998; Riera-Lizarazu et al., 2000). Chromosomal rearrangements induced by irradiation are usually non-compensating and may result in genomic instability in the subsequent generations. Nevertheless, irradiation methods have some practical advantages over recombination-mediated strategies: (*i*) theoretically, any alien chromosome segment could become inserted into a wheat chromosome, (*ii*) the efficiency with which interspecific translocations are formed is not affected by the pairing potential between alien and wheat chromosomes (Jiang et al., 1994). The irradiation of wheat–alien addition or substitution lines may thus yield a large number of intergenomic translocations between the alien and wheat genomes. The selfing or backcrossing of the irradiated generation may result in a large range of translocation lines. These lines can be selected for favourable alien traits and then introduced into breeding programmes. Before applying such a breeding strategy, it is important to investigate the frequency and transmission pattern of the chromosomal aberration types generated between wheat and alien chromosomes.

Sensitive molecular cytogenetic and molecular genetic methods help to analyse wheat–alien introgression lines. Genomic *in situ* hybridization (GISH) (Reader et al., 1994) provides a direct, visual method for detecting alien chromatin introgressed into the wheat genome (Schwarzacher et al., 1989; Le et al., 1989; Mukai and Gill, 1991).

Earlier, five wheat–barley translocations were produced in Martonvásár using tissue culture (Molnár-Láng et al., 2002). Recently, the development of a new winter wheat–winter barley disomic addition set (Martonvásári 9 *krl*–Igri) was reported by Szakács and Molnár-Láng (2007; 2010). This addition set was used to investigate the formation of intergenomic chromosome rearrangements between wheat and barley genomes induced by gamma irradiation.

Materials and methods

Plant materials

Wheat–barley disomic addition lines were selected in Martonvásár from the backcrossed and selfed progenies of a hybrid produced by Molnár-Láng et al. (2000b) using the Hungarian winter wheat (*Triticum aestivum* L., $2n = 6x = 42$) line Martonvásári 9 *kr1* (Mv9kr1) (Molnár-Láng et al., 1996) as maternal parent and the 2-rowed winter barley (*Hordeum vulgare* L., $2n = 2x = 14$) cultivar Igri as the male parent, and identified using *in situ* hybridization methods (Szakács and Molnár-Láng, 2007; 2010). To study genetic stability (that is, what percentage of the plants retained the disomic state in the next generation), 50 seeds from each line (containing 44 chromosomes) of the addition set were selected. Chromosome composition was determined in root cells using GISH. Chromosome breakages were induced in dry seeds of the addition lines using irradiation with ^{60}Co gamma rays at a dosage of 50 Gy. The mutagenized generation (M_0) was analysed by GISH to detect intergenomic rearrangements.

Genomic in situ *hybridization*

Root-tip metaphase chromosome preparations were made from germinating seeds of each addition line following the method described by Jiang et al. (1994). GISH was carried out according to Reader et al. (1994) with minor modifications (Molnár-Láng et al., 2000a). Total barley genomic DNA was labelled with Fluorored by nick translation (Nick Translation Mix, Roche) and used as a probe. Unlabelled wheat genomic DNA was sheared by autoclaving and used as blocking DNA at 30× the quantity of the probe. Each denatured slide was loaded with 50 µl of denatured hybridization solution containing 2 × SSC, 10% dextran sulphate, 0.2% SDS and 1 ng/µl labelled probe DNA together with the blocking DNA and incubated for 2.5 hours at 65°C. Stringency washing was carried out in 2 × SSC at 42°C for 2 × 5 min. The slides were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Amersham) and mounted in Vectashield antifade solution (Vector Laboratories). Fluorescent signals were visualized with a Zeiss Axioscope 2 epifluorescence microscope fitted with a Spot CCD camera (Diagnostic Instruments, Inc., USA). The image processing was carried out using Image-Pro Plus 5.1 software (Media Cybernetics, USA).

Results and discussion

The high stability of addition lines is an important criterion for applying them in chromosome manipulation techniques. The genetic stability of the Mv9krl–Igri disomic addition lines was analysed in root-tip preparations made from the 1HS isochromosomic and 2H, 3H, 4H, 6HS and 7H disomic addition lines. The results are summarized in Table 1. The most stable addition lines were the 2H and 3H disomic additions, where all the progeny plants contained 44 chromosomes. This is in contrast with the observation of Islam et al. (1981) that none of the Chinese Spring–Betzes addition lines were completely stable. The rate of disomic 2H progenies was 91.6% in the Chinese Spring–Betzes addition line and 88.9% in another 2H addition line (Linc and Molnár-Láng, 2003). The 7H disomic and 6HS ditelosomic addition lines can also be regarded as stable, as 96.4% and 90.0% of their progenies remained in the disomic state, respectively, and the total elimination of the barley chromosomes did not occur. Similar rates of 94.6% for the 7H and 93.5% for the disomic 6H were found in Chinese Spring–Betzes addition lines. The stability of the 6H disomics (96.1%) reported

by Linc and Molnár-Láng (2003) was nearly the same, but the 6HS ditelosomic addition line described by Molnár-Láng et al. (1996) showed a stability of only 50.0%. The Martonvásári 9krl–Igri 4H addition line also exhibited good stability. Despite the elimination of barley chromosomes from the progenies (21.1%), nearly 80% of the plants remained in the disomic or monosomic state.

A fertile disomic addition line involving the entire barley chromosome 1H cannot be produced because of the *Shw* sterility gene present on the long arm. The addition line disomic for the 1HS isochromosome is self-fertile, but unstable. The occurrence of the full pair of 1HS isochromosomes was only 5.7% in the next generation after selfing. However, the barley 1HS isochromosomes were retained in the monosomic state or as telocentric chromosomes in 62.9% of the progeny plants. This indicates that the Mv9krl–Igri disomic addition lines are stable enough to be used as starting material for producing translocations.

Table 1
Stability of the Martonvásári 9 krl–Igri disomic addition set (W - wheat, B - barley)

Addition lines		Chromosome composition of progeny plants (%)		
		42W+2B	42W+1B	42W
1HS isochromosomic		5.7	22.9	31.4
2H disomic		100.0	–	–
3H disomic		100.0	–	–
4H disomic		68.4	10.5	21.1
6HS ditelosomic		90.0	10.0	–
7H disomic		96.4	3.6	–

Chromosome rearrangements were induced by gamma irradiation to create intermediate materials valuable for the transfer of agronomically valuable traits from barley to bread wheat. The M_0 generation of wheat–barley addition lines was analysed by GISH to determine the frequency of intergenomic chromosomal changes between the two genera. Using labelled total genomic DNA, barley chromatin could be clearly discriminated from the unlabelled wheat chromosomes. Irradiation was 100% effective, as chromosome rearrangements could be detected between the two genomes in every plant of the mutagenized generation. The irradiation-induced chromosomal aberrations showed a mosaic pattern, i.e. cells of the same plant carried different types of translocation chromosomes (Fig. 1). The frequency of chromosomal aberrations was compared in 42 cells of a single plant and in 324 cells of 15 plants. The data summarized in Table 2 show that reciprocal and interstitial translocations were the most frequent aberrations (about 30–40%) in both cases. Terminal translocations were observed at a frequency of about 25%, while centric fusions were formed in less than 10% of the cells.

Table 2

Number and frequency of different types of translocations in the M_0 generation of the Martonvásári 9 kr–Igri disomic addition lines

Types of translocation	1 plant, 42 cells		15 plants, 324 cells	
	No.	%	No.	%
Centric fusions	2	4.2	39	9.0
Terminal	11	22.9	111	25.6
Interstitial	19	39.6	128	29.6
Reciprocal	16	33.3	155	35.8

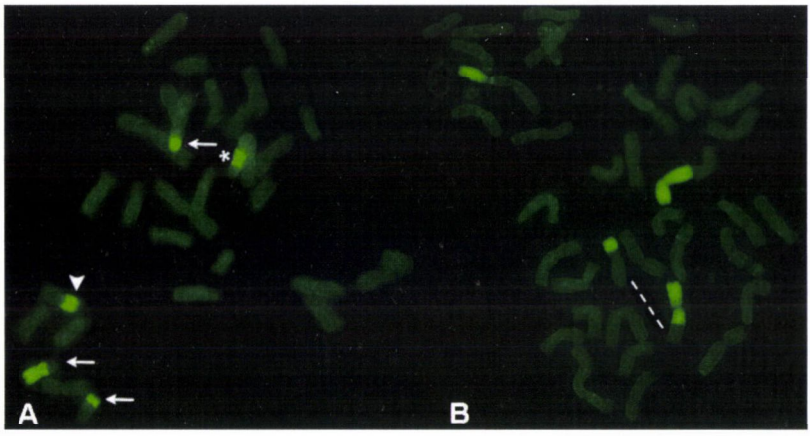

Fig. 1. Detection of barley chromosome segments by GISH in partial metaphase cells of a gamma-irradiated addition line. The chromosomes were hybridized with total barley genomic DNA labelled with Fluorored. Terminal translocations (arrows), a centric fusion (arrowhead) and an interstitial translocation (asterisk) (A), and chromosomes with a reciprocal translocation (dotted line) (B) can be distinguished in two cells of a single plant

The relatively high frequency of interstitial chromosome rearrangements is surprising. In general, terminal translocations and centric fusions are more abundant than other types of translocations (Friebe et al., 1996; Badaeva et al., 2007), as also found in a recent study on gamma-irradiated wheat–*Ae. biuncialis* amphiploids (Molnár et al., 2009). This is explained by the fact that insertions need a minimum of two breakpoints in one of the affected chromosomes, which is a much rarer event than the breakage pattern needed for reciprocal or terminal translocations (Sybenga, 1992). Nevertheless, the leaf rust (*Puccinia recondita* Rob. ex Desm.) resistant line isolated by Sears (1956) from irradiated addition lines possessed an intercalary translocation in which a piece of *Aegilops umbellulata* chromatin, carrying the factor for resistance, had been inserted into a wheat chromosome. Friebe et al. (1996) also identified two intercalary translocations in wheat–alien introgression lines, conferring resistance to diseases.

The selfed M_1 generation will be studied to evaluate the transmission of irradiation-induced chromosomal alterations. Translocations produced in this way are expected to be stabilized in later backcross progenies as a set of introgression lines carrying few but distinct rearrangements, which is planned to be characterized using cytomolecular tools.

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CHARACTERIZATION OF WHEAT–BARLEY INTROGRESSION LINES FOR DROUGHT TOLERANCE

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The safety of wheat production in Hungary requires the propagation of drought-tolerant cultivars because of the regular occurrence of water deficiency. Hybridization between related species makes it possible to transfer desirable traits from one species to another. Introgression lines developed from wheat/barley hybrids were investigated together with the parental wheat and barley cultivars to determine how the added barley chromosome (segment) influences drought tolerance in wheat. The plants were grown in the field at the UP Georgikon Faculty, Keszthely. Sowing and harvest were done by hand. Half the length of the 12 m rows was covered with a plastic rain shelter on 2nd April (EC: 30–31) to protect the plants from rain, resulting in a 163 mm difference in water supplies between the control (not covered) and stressed (covered) treatments. Data were obtained for anthesis and maturity date, plant height, root/shoot ratio, leaf water potential, grain yield and grain yield components. The plants adapted to water deficiency by increasing the root/shoot ratio and decreasing the water potential and the duration of grain filling. The grain yield was reduced by 12%, averaged over the genotypes, mainly due to a decrease in the number of spikes per plant.

Key words: wheat–barley hybrids, drought stress, water potential, yield, yield components

Introduction

The safety of wheat production in Hungary is often endangered by drought, so the efficient use of the water stored in the soil has special significance. Global climate change is likely to increase the area of drought-prone land in the future, as annual precipitation may decrease, while its spatial and time distribution will be even less favourable (Reynolds et al., 2007; Várallyay, 2008). This will require the propagation of drought-tolerant cultivars.

Drought is one of the most important environmental stresses. The reduction in grain yield depends not only on the duration and intensity of water stress, but also on the developmental phase at which the stress was imposed

(Samarah et al., 2009). Numerous physiological and morphological changes occur in plants in response to drought stress, including a reduction in water potential (Mogensen, 1992; Forster, 2004) and a change in the root-shoot ratio.

Hybridisation between related species makes it possible to transfer desirable traits from one species to another (Molnár et al., 2007). Barley, known to have good drought tolerance, is a potential gene source for wheat improvement. The introgression of barley (*Hordeum vulgare* L.) chromosome segments into wheat (*Triticum aestivum* L.) may result in the transfer of new, stress-adaptive traits, such as earliness, and tolerance of drought or soil salinity into wheat.

Since the first successful hybridisation between wheat and barley (Kruse, 1973) only a few wheat–barley translocation and substitution lines have been developed (Islam and Shepherd, 1992; Koba et al., 1997; Molnár-Láng et al., 2000). These wheat–barley hybrids were only investigated for cytogenetic characteristics and fertility. Very little information is available on the ability of barley chromosomes to compensate for wheat chromosomes in terms of agronomically important characteristics in plants grown in hydroculture (Molnár et al., 2007) and no information is available on the behaviour pattern of wheat–barley derivatives grown in the field.

The aim of this study was to determine how added barley chromosomes (segments) influence various agronomic traits, especially drought tolerance in wheat.

Materials and methods

Experimental conditions

The field experiment was carried out at the UP Georgikon Faculty, Keszthely during the 2008–2009 season. Each genotype was sown in a 15-m row, with a row spacing of 25 cm. Half of each row was covered with a plastic rain shelter on 2nd April (EC: 30–31) to protect the plants from rain, resulting in a 163 mm difference in water supplies between the control (not covered) and stressed (covered) treatments. The 50-year annual average rainfall (1951–2000) in Keszthely was 653 mm, while the total rainfall during the growing season in the present experiment was 471 mm. The soil of the experimental site is a lessivated brown forest soil (FAO: Luvic phaosem) with low organic material and medium K and P contents. Weeds were removed by hand throughout the experiment. Sowing and harvest were done by hand.

Data were obtained for the dates of flowering and maturity, plant height, root/shoot ratio at EC 30–31, leaf water potential Ψ_L, determined in a pressure chamber (PMS Instrument) using N_2 gas, ear length, number of kernels per ear, thousand-grain weight and grain yield.

Plant material

Wheat/barley addition, substitution and translocation lines were developed in Martonvásár by hybridising various wheat and barley cultivars. The wheat/barley disomic addition lines 2H, 3H and 4H and the 7DL.7DS-5HS translocation were developed from the hybrid combination Mv9 kr1 (Martonvásár winter wheat genotype) × Igri (German two-rowed winter barley). The translocations 2DS.2DL-1HS, 3BL.3HS and 6BS.6BL-4HL and the substitution 4H(4D) originated from the cross (Chinese Spring spring wheat × Betzes spring barley) × Mv9 kr1, while 4H(AsMa) was developed from the hybrid combination Asakaze komugi (Japanese wheat) × Manas (Ukrainan six-rowed winter barley). The barley chromosomes were detected using genomic *in situ* hybridisation (GISH) and identified with the help of fluorescence *in situ* hybridisation (FISH) with repetitive DNA probes.

Results

Root/shoot ratio

From the point of view of drought tolerance, root length and weight and the root/shoot ratio are important characteristics. At tillering (EC 30–31) six plants of each genotype were dug up. The length and dry mass of the roots and shoots were measured and the root/shoot ratio was calculated (Fig. 1). The root/shoot ratio of wheat–barley derivatives varied from 39–64%, while that of the parents was 43 and 21% for Mv9 kr1 and Igri, respectively. The highest root/shoot ratio was found for 3H and 2DS.2DL-1HS (64 and 63%) but these values were based on very different levels of root and shoot dry weight (3H: 3.22 and 5.03 g; 2DS.2DL-1HS: 0.57 and 0.90 g root and shoot dry weight, respectively). Although a higher root/shoot ratio may contribute to increased drought tolerance, the root biomass must also be taken into consideration.

Date of flowering and maturity

Water deficiency resulted in earlier flowering (3 days for the wheat parent, 1 day for the barley cultivars and 1 or 2 days for the lines). The spikes also matured earlier (Figs. 2 and 3), resulting in a shortening of the grain-filling period. The difference between the control and stressed plants was 3 and 4 days for the parental cultivars and 6 days for most of the lines, but there was no shortening of the grain-filling period in the case of lines 2H (Igri) and 4H (Manas), while the difference between the control and stressed plants increased to 7 days for 4H (Igri).

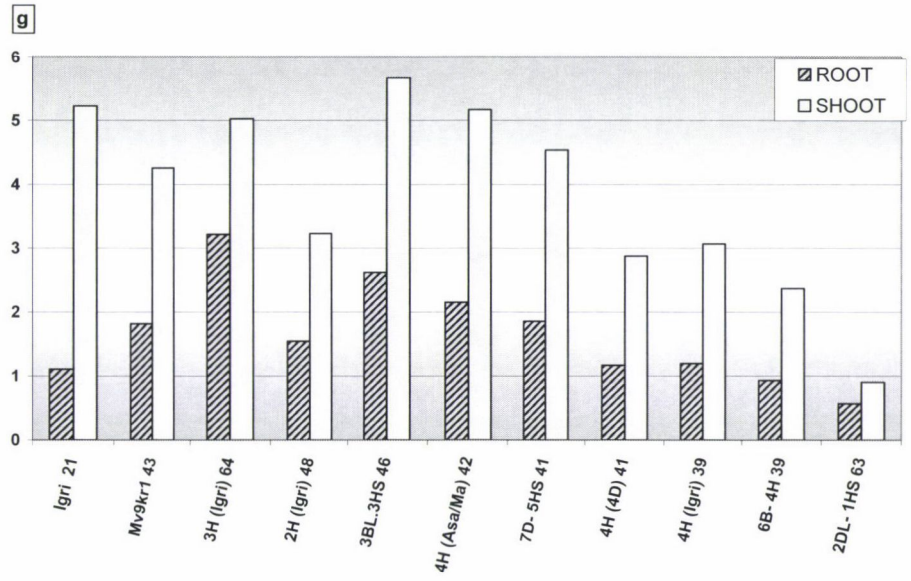

Fig. 1. Root and shoot dry weight (g) and root/shoot ratio (given after the line code) of wheat–barley derivatives and the parental cultivars

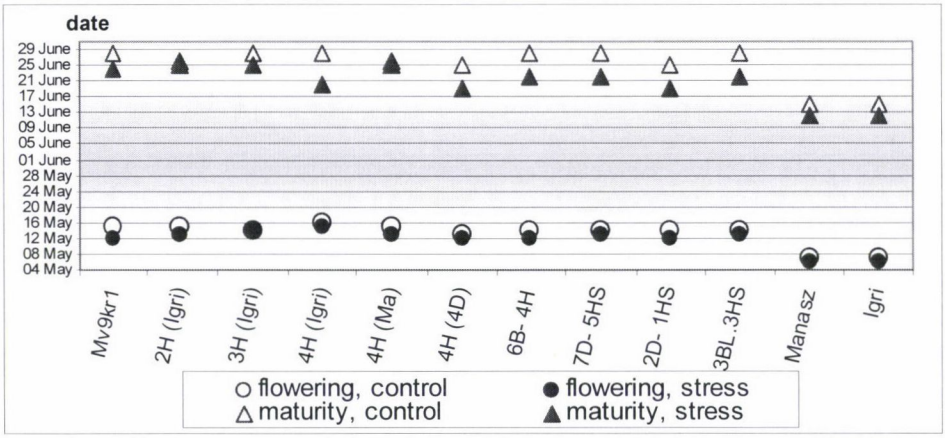

Fig. 2. Dates of 50% flowering and maturity for wheat–barley derivatives and their parents in the stress and control treatments

Fig. 3. Introgression lines developed from field-grown wheat/barley hybrids and the parental cultivars, where half of each 12-m row was covered with a plastic tent to protect plants from rain in the stress treatment

Water potential

During drought stress the water potential (Ψ_L) of the plant decreases, leading to the strengthening of the suction force, which can be considered to be the result of osmotic adaptation, the drought tolerance strategy of the genotype. In the present experiment the leaf water potential in the stress treatment was reduced by 24%, averaged over the genotypes (Fig. 4). The water potential of

Igri only decreased by 3%, but a highly negative value (–1.86 Mpa) was also measured in the control treatment for this cultivar. The greatest decrease in water potential (49%) was measured for 2DS.2DL-1HS, while 2H, 4H(Ma), 7D-5HS and 3BL.3HS exhibited similar reductions in water (32–35%).

Plant height

All the plants from the middle 1 m of each row under the rain shelter and in the control (uncovered) part were hand harvested. Plant height was measured from the soil surface to the top of the spike excluding the awns. The number of spikes per plant, number of grains per spike, 1000-grain weight and total grain yield were measured after hand threshing. The plant height of the parental wheat and barley cultivars was fairly similar (84–95 cm), but the height of the hybrid combinations varied from 70 to 122 cm, and there was one dwarf (43 cm) line as well: 2DS.2DL-1HS (Table 1, Fig. 3).

Grain yield and yield components

The grain yields recorded for the wheat–barley derivatives and their parents are shown in Table 1. The grain yield was reduced by 12%, averaged over the genotypes, in the stressed treatment. The barley parent Igri had a yield loss of 10% and the wheat parent Mv9kr1 11%. The highest yield loss was measured in the case of 6B-4H (25%), while no yield decrease was observed for 4H(4D). Drought susceptibility is often calculated on the basis of yield reduction, but the absolute values also need to be taken into consideration. In spite of the 25% yield decrease, 6B-4H yielded twice as much as 4H(4D) in the stress treatment. Among the wheat–barley derivatives, 2DS.2DL-1HS and 4H(4D) had the smallest yield, while 3BL.3HS and 7DL.7DS-5HS had the highest yield in both treatments.

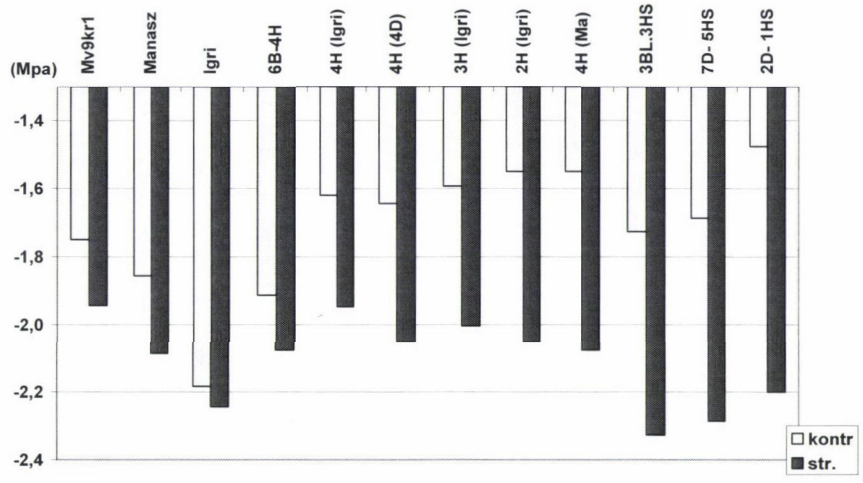

Fig. 4. Leaf water potential (Ψ_L) of wheat–barley derivatives and their parents in the control and stress treatments measured on the flag leaf at midday

Table 1

Plant height (cm), yield and yield components of wheat–barley derivatives and the parent cultivars grown in the field under a rain shelter (stressed) and under rain-fed (control) conditions during the 2008–2009 season

Geno-type	Plant height	Spikes per plant (n)			Grains per spikes (n)			1000-grain weight (g)			Grain yield (g m^{-1})		
		control	stress	%	control	stress	%	control	stress	%	control	stress	%
Mv9kr1	84	3.0	2.8	94	54.0	50.1	93	46.4	47.9	103	204.4	181.6	89
Manas	95	3.6	3.3	91	47.1	48.0	102	51.6	49.9	97	196.4	181.1	92
Igri	88	5.8	5.3	92	26.0	25.2	97	57.1	57.1	100	245.0	220.0	90
2H	76	2.7	2.1	78	38.6	44.0	114	43.1	42.2	98	95.7	83.4	87
3H	83	3.0	2.6	87	34.0	34.0	100	42.5	44.8	105	126.5	115.0	91
4H	70	2.0	2.1	104	48.1	45.0	94	33.7	32.8	97	97.8	92.9	95
4H(Ma)	116	3.6	3.4	94	31.0	32.0	103	35.2	34.1	97	129.0	121.1	94
4H(4D)	77	3.4	2.8	82	27.5	33.4	121	35.4	36.3	102	54.3	55.4	102
6B-4H	105	3.1	2.1	69	34.6	36.0	104	35.2	37.0	105	146.0	109.5	75
7D-5HS	74	3.6	3.1	86	37.0	36.0	97	41.5	42.0	101	181.3	152.0	84
2D-1HS	43	2.3	1.9	83	36.4	38.0	104	28.9	27.3	94	37.3	30.3	81
3BL.3HS	122	3.1	3.0	98	43.4	37.0	85	43.7	42.9	98	215.0	172.0	80
Mean	*86*	*3*	*3*	*88*	*38*	*38*	*101*	*41*	*41*	*100*	*144*	*126*	*88*

Yield loss was caused by a decrease in the number of spikes (Table 1), while the grain number per spike and the 1000-grain weight did not change, averaged over the genotypes. The only exception was line 3BL.3HS, where the yield loss originated from a reduction in the number of grains per spike.

Discussion

The consequences of drought stress for morphological and agronomical traits were investigated on introgression lines developed from wheat/barley hybrids and the parental wheat and barley cultivars during the 2008–2009 season. Drought stress was triggered by a plastic rain shelter, which caused 163 mm difference in the water supplies between the control (not covered) and stressed (covered) treatments.

Although a larger root-shoot ratio could contribute to increased drought tolerance (Hoffmann, 2008), the absolute values of root and shoot weight must also be considered, since a promising root-shoot ratio may cover very low root and shoot biomass, as in the case of line 2DS.2DL-1HS.

In the stress treatment the plants matured earlier than in the control treatment, resulting in a shorter grain-filling period. According to Sanchez et al. (2002) the negative effect of drought was due to a reduction of 5 days in the duration of grain-filling in barley. The results reported by Asseng and Herwaarden (2003) confirm that water stress during the grain filling period induces early senescence and shortens the duration of grain filling.

Osmotic adaptation caused by water deficiency results in a decrease in water potential, which leads to the strengthening of the suction force. Savin and

Nicolas (1999) found that the leaf water potential of barley decreased to –2.5 MPa after drought stress during the-grain filling period. Similar values were measured in the present experiment: the leaf water potential was between –1.94 and –2.33 MPa in the stress treatment, equivalent to a decrease of 24% compared with the control plants, averaged over the genotypes. The lower water potential during drought stress can be explained by the decrease in water content and the increase in the concentration of salts and ABA in plant cells (Nayyar and Walia, 2004).

Drought susceptibility is often calculated on the basis of the yield reduction caused by water deficiency. In the present experiment the greatest yield loss was measured in lines 6B-4H and 3BL.3HS (25 and 20%, respectively), but if the absolute yields in the stress treatment are considered, it can be seen that in spite of the yield decrease, 6B-4H yielded twice as much as 4H(4D), which suffered no loss of yield. Drought stress reduces the grain yield by decreasing the number of grains or the individual grain weight (Samarah, 2004). In the present experiment differences in grain yield were related to spike number per plant rather than grain number per spike.

These data provide information on how the added barley chromosomes (segments) influence various agronomic traits, especially drought tolerance in wheat.

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EFFECT OF DROUGHT STRESS AT FLOWERING ON THE WATER POTENTIAL AND PHOTOCHEMICAL REACTIONS OF RECIPROCAL MAIZE HYBRIDS

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The research, carried out in the Martonvásár phytotron in 2007, was aimed at determining how the leaf water potential of maize hybrids produced in direct and reciprocal crosses, and thus possessing different levels of seed vigour, changed as the result of water withholding in the flowering phenophase. In the case of the silage maize hybrids Mv 290 and Lima it was found that seedling vigour influenced the plant height (measured at 30 days) of adult plants. Crosses produced on chilling-sensitive female genotypes (GL, AM, H29), such as the hybrids Káma, Maraton and Hunor, proved to be unambiguously stress-sensitive if water was withheld for more than six days. In all cases drought stress reduced the relative quantum efficiency, irrespective of the crossing combination.

Key words: seed vigour, water withholding

Introduction

In 2007 Hungary had a taste of the unfavourable consequences of global warming. The present work investigated the global warming factors most important for agriculture, and particularly for hybrid maize, the crop grown on between a quarter and a third of the arable land. In 2007, as previously experienced in 1992, 2002 and 2003, a sudden rise in temperature in spring led to the rapid drying of the soil, leading to a reduction in water capacity and the deterioration of germination and emergence conditions, while the drastic summer heat (with ten consecutive days of extremely high temperatures) not only reduced pollen viability, but also caused a decline in the moisture potential of the silks, making even a low level of fertilisation difficult. Hybrid maize seed production suffered enormous losses, with yields of only 0.2–0.3 t/ha in places, which was only a fraction of the 1.0–1.2 t/ha average. The situation was further complicated by poor stands and flowering abnormalities.

Drought tolerance is thus a key factor, as reproductive processes require a moist environment. Plants that survive unfavourable conditions in the initial stages of development (Goulas et al., 2000; Toma et al., 2000; Jacota and Visnevski, 2000; Vujakovic et al., 2000) still require a satisfactory moisture content in the flowering phenophase (foliage, ear-leaf). Pollen is the driest plant organ, so the silks must retain water to ensure a favourable environment for pollen germination and pollen tube growth (Herrero, 1980; Dupuis and Dumas, 1990). High temperatures and desiccation may cause anomalies in pollen shedding and pollen viability, and may shorten the period when the stigma is receptive for fertilisation (Westgate and Boyer, 1986; Zinselmeier et al., 1990; Aluchi, 2000).

Previous observations suggested that male sterility could be induced not only by genetic factors, but also by drought and heat stress (Palágyi, 1975). Flowering asynchrony may result in both reduced pollen viability and embryo abortion (Freier et al., 1984; Zinselmeier et al., 1990; Quarrie et al., 2000), reported by other authors as intolerance of desiccation (Kollipara et al., 2002).

In response to drought stress, changes also take place in chlorophyll fluorescence induction parameters (Janda et al., 1994; Berzy et al., 1998). In addition to drought, the genetic composition of the hybrids, which determines their resistance, may also be decisive for quantum efficiency and photochemical quenching (Berzy et al., 1998).

Many authors have suggested a correlation between seed vigour and the sensitivity of maize hybrids to environmental stress (Lakshmi et al., 2001; Xu et al., 2003; Veselova and Veselovsky, 2000; Berzy et al., 1998). The stress resistance of the female genotype (Berzy et al., 2007) may also be based on the soil depth from which the seedling, and later the adult plant, is able to absorb the necessary water. The root mass may thus also be correlated with the grain yield (Sanguinetti et al., 2000; Grzesiak, 2001).

The present experiments aimed to investigate how the moisture potential of the canopy varied in response to different extents of water withholding in plant stands developing from seed originating from direct and reciprocal crosses. According to Biasutti and Galinanes (2001) there is no correlation between germination stress, which determines the biological value of the seed, and the grain yield of adult plants, but Tonin et al. (2000) and Lakshmi et al. (2001) reported contradictory results. In order to clarify the situation, studies were made on correlations between the seed vigour of maize hybrids bred in Hungary and the stress resistance and photochemical responses of seedlings and adult plants.

Materials and methods

An experiment involving water withholding was set up in the phytotron of the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, in 2007. The hybrids included in the experiment, namely Káma (GL × H 05), Lima (GL × H 41), Gamma (LJH × H 05), Mv 290 (H 26 × H 05), Hunor (H 22 × H 29), Maraton (AM × H 05) and their reciprocal crosses,

were selected on the basis of a complex seed vigour test (96 h hypoxia, 48 h chilling at 5°C), which expresses the biological value of the seed by mimicking environmental stress conditions after sowing in a cool wet spring (Barla-Szabó and Berzy, 1989). After germination for 14 days in a Fitoclima germination chamber at 25°C and 75% relative humidity (Perry, 1981) the most vigorous seeds of each cross were planted four to a pot in four replications in 10-litre pots containing a 3:1 mixture of soil and sand and placed in a PGV 72 chamber under the climatic conditions reported by Berzy et al. (1998), except that in the third week the climatic program was modified to reflect the adverse weather conditions to which maize plants are exposed in the case of early sowing (especially on the Great Hungarian Plain). After the fourth week a day/night temperature of 30°/20°C was programmed, with a 14-h daylength. The illumination was provided by metal halide lamps with a light intensity of 250 μmol/m²/s.

Plant height was scored when the plants were 30 days old, after which the number of plants per pot was reduced to two, leaving only the most vigorous plants. All the pots were irrigated equally until the tasselling phenophase. One pot per genotype was then designated as an irrigated control, and water was withheld from the other three pots to represent drought stress. On the 4th, 6th, 8th and 10th day of drought treatment two 6×3 cm samples, cut from the leaf next to the ear primordium, were taken from stressed plants of each cross. The samples were examined with a hand-held moisture potential meter (PMS, Oregon, USA). Due to the smaller number of plants, only one sample was taken from each control treatment.

The effect of drought on maize plants was only examined at flowering, since the most sensitive time from the point of view of seed yield is the period before and during flowering. The soil moisture content was not measured because of the small capacity of the pots.

Chlorophyll-a fluorescence induction measurements

The chlorophyll fluorescence induction parameters of the maize leaves were determined at ambient temperature using a pulse amplitude modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany) as described earlier (Janda et al., 1994). Before the measurements, the plants were dark-adapted for 30 min. A built-in halogen lamp (PPFD = 200 μmol m^{-2} s^{-1}) was used for actinic illumination.

Statistical analysis

The results were evaluated for each hybrid using single-factor analysis of variance. A two-sample t-test was used to analyse the fluorescence induction parameters.

Results and discussion

Effect of drought stress on the vigour and leaf water potential of reciprocal maize hybrids

In the case of the early silage hybrid Lima, the seed vigour, symbolising the biological value of the seed, exhibited considerable differences depending on whether the inbred line GL, which carries the *leafy* gene, was used as the male or female partner (Table 1). GL is a chilling-sensitive line with low vigour, which transmits its poorer vigour when used as female partner in crossing combinations. The 30-day-old plants were more than 10 cm shorter than when the Lima hybrid was produced using H 41 as the female partner. During the first part of the drought treatment a gradual decline in water potential was observed, but after the 8th day the damage appeared to be irreversible when GL was used as female partner (Table 1).

Table 1

Effect of drought stress on the seed vigour, leaf water potential [LWP (MPa) after 4, 6, 8 or 10 days of water withholding at tasselling], pollen production and plant height of direct and reciprocal crosses of the maize hybrid Lima (Martonvásár, 2007)

Genotype	Vigour (%)	LWP				Pollen (g/tassel)	Plant height at 30 d (cm)
		4 days	6 days	8 days	10 days		
Lima							
GL♀×H41♂ S	51.5*	–0.54	–0.80	–0.95	–1.15		27.09*
GL♀×H41♂ C		–0.06	–0.05	–0.05	–0.07	0.0935*	
H41♀×GL♂ S	79.0	–0.30	–0.40	–0.62	–0.75		39.25
H41♀×GL♂ C		–0.08	–0.07	–0.08	–0.05	0.0032	
$LSD_{5\%}$	12.8		0.43			0.044	10.6

*Vigour: assessed using the complex stressing vigour test (Barla-Szabó and Berzy, 1989); S: stressed plants; C: control plants

As in the case of Lima, both the seeds and 30-day-old plants of the silage maize hybrid Káma were more vigorous when GL was used as the male partner. The high water potential values observed at the beginning of drought stress (on the 4th day) decreased drastically after the 6th day in the GL × H 05 cross, while in the reciprocal cross the initial leaf water potential was lower, but water was lost more slowly and evenly, suggesting that these plants had better stress tolerance.

When the grain maize hybrid Gamma was produced on line LJH, which had poorer physiological seed vigour, the plant height at 30 days of age was very similar to that of the reciprocal cross under favourable environmental conditions, despite the weaker vigour (Table 1). After six or more days of water withholding, however, the sensitivity of LJH became more apparent, like that of GL. In the reciprocal cross (H 05 × LJH) the plants exhibited a reliable level of stress tolerance.

The registered hybrid Mv 290 is a single-cross grain maize developed from a cross between early (H 26) and mid-late (H 05) inbred lines. In the form H 26 × H 05 the seed had poorer vigour and the plants were thinner and much shorter than those of the reciprocal cross (Table 1). The poorer biological value of the seed was not reflected, however, in the drought tolerance of the adult plants, as the leaf water potential of the H 26 × H 05 combination did not decline until the 6th day. This phenomenon should be tested on a larger number of plants in the field.

The mid-season hybrid Hunor SC (H 29 × H 22) owes its poor vigour to the chilling sensitivity of the H 29 line (Table 1). The reciprocal form, with H 22 as the female parent, is far more tolerant of unfavourable environmental conditions in the initial stages of development. The plant height at 30 days, however, revealed no significant difference between the two combinations. The leaf water potential of H 29 declined greatly after six days of water withholding (Table 1), but this reduced level was then retained to the end of the treatment. Plants from the H 22 × H 29 cross had good initial tolerance of water deficiency, the first signs of drought sensitivity being observed after ten days.

As in the case of Káma and Gamma, plants of the late (FAO 500) hybrid Maraton SC had good drought tolerance when grown from seed developed on inbred line H 05. When the less vigorous line AM was used as the female parent, the initial leaf water potential was lower, but after 10 days of drought, plants of both crossing combinations exhibited irreversible wilting. The lowest leaf water potential values in the experiment were recorded for this hybrid, underlining the need to grow it under irrigated conditions.

Due to the rudimentary and deformed nature of the tassels on stressed plants, pollen grain fertility could only be examined for the control plants. Genotypes with H 22 and H 05 as female parent (hybrids Gamma and Hunor) were not only more drought-tolerant, but also had significantly greater pollen-supplying ability than the reciprocal forms (Table 1). The hybrid Lima also produced more pollen when developed on the GL inbred line than when H 41 was used as female parent.

The leaf water potential at flowering declined as the result of water withholding to varying extents in all the genotypes, but the reduction was not always drastic (Aluchi, 2000). Very few authors have reported a correlation between seed biological value, seedling parameters and the grain yield (Lakshmi et al., 2001; Berzy et al., 2007), while none was found by Biasutti and Galinanes (2001).

There was a clear correlation between vigour (based on seedling mass and length) and drought tolerance in the present work for genotypes GL and LJH. Crosses made on female plants classified as chilling-sensitive on the basis of seed analysis all proved to be drought-sensitive when water was withheld for more than six days. These laboratory results require confirmation in the field.

The poorer seed vigour when hybrids were developed on stress-sensitive female plants was evident in the plant height (hybrids Káma, Lima, Mv 290), while – on the basis of drought tolerance data – the inbred line LJH was noted for its poorer physiological seed vigour and for the rapid decrease in leaf water potential when the line was used as female crossing partner (hybrid Gamma).

Effect of drought stress on the photochemical responses of reciprocal maize hybrids

The effect of water withholding on the functioning of the photosynthetic apparatus was characterised by chlorophyll-*a* fluorescence induction on the 8th day of treatment. In response to drought stress there was generally a slight reduction in the maximum quantum efficiency (Fv/Fm) and a greater decline in the effective quantum yield (ΔF/Fm') and photochemical quenching (qP), while the non-photochemical quenching (qN) increased. These changes were dependent on the genotype. In the case of hybrids Lima, Káma, Hunor and Maraton, drought stress caused a significant reduction in Fv/Fm in both crossing combinations (Table 2). For Mv 290 the reduction was not significant for either combination, while for Gamma it was not significant for the LJH × H 05 form. The ΔF/Fm' parameter is generally more sensitive than Fv/Fm, declining earlier and to a greater extent. Judging from the quantum efficiency and photochemical

quenching parameters, only the direct and reciprocal combinations of the hybrid Maraton give an unambiguous response to water withholding. For both parameters the control plants significantly surpassed the stressed plants. In many cases where the effect of drought stress was not significant, the low control value may have been the reason (e.g. GL × H 41, LJH × H 05). According to seed vigour analysis, crosses produced on chilling-sensitive female lines (GL × H 05, AM × H 05, H 29 × H 22) proved to be stress-sensitive on the basis of all the photochemical parameters after six days of water withholding (Table 2).

It is clear from the results that stress-sensitive hybrids should always be produced on the more vigorous female line.

Table 2
Effect of 6 days of water withholding on the chlorophyll-*a* fluorescence induction parameters of reciprocal maize hybrids

Genotype	Treatment	Fv/Fm	ΔF/Fm'	qP	qN
Lima					
GL×H41	Control	0.761 (±0.016)	0.251 (±0.125)	0.506 (±0.221)	0.688 (±0.054)
	Drought	0.613 (±0.050)*	0.146 (±0.014)	0.483 (±0.049)	0.783 (±0.042)
H41×GL	Control	0.774 (±0.019)	0.406 (±0.131)	0.636 (±0.155)	0.428 (±0.131)
	Drought	0.673 (±0.017)*	0.146 (±0.011)*	0.457 (±0.070)	0.770 (±0.054)*
Káma					
GL×H05	Control	0.782 (±0.007)	0.489 (±0.072)	0.790 (±0.051)	0.489 (±0.122)
	Drought	0.604 (±0.066)*	0.148 (±0.033)*	0.575 (±0.043)*	0.849 (±0.075)*
H05×GL	Control	0.771 (±0.009)	0.341 (±0.143)	0.605 (±0.170)	0.605 (±0.158)
	Drought	0.666 (±0.031)*	0.162 (±0.033)	0.524 (±0.083)	0.815 (±0.033)
Gamma					
LJH×H05	Control	0.737 (±0.015)	0.244 (±0.100)	0.406 (±0.118)	0.394 (±0.191)
	Drought	0.723 (±0.050)	0.158 (±0.015)	0.563 (±0.042)	0.868 (±0.014)*
H05×LJH	Control	0.756 (±0.015)	0.336 (±0.120)	0.604 (±0.176)	0.577 (±0.116)
	Drought	0.651 (±0.054)*	0.153 (±0.023)	0.471 (±0.088)	0.766 (±0.036)
Mv 290					
H26×H05	Control	0.773 (±0.012)	0.352 (±0.180)	0.564 (±0.262)	0.428 (±0.119)
	Drought	0.767 (±0.011)	0.230 (±0.042)	0.440 (±0.038)	0.531 (±0.070)
H05×H26	Control	0.779 (±0.016)	0.464 (±0.043)	0.730 (±0.051)	0.437 (±0.079)
	Drought	0.721 (±0.034)	0.191 (±0.089)*	0.490 (±0.086)*	0.684 (±0.185)
Hunor					
H29×H22	Control	0.803 (±0.007)	0.471 (±0.047)	0.761 (±0.054)	0.553 (±0.048)
	Drought	0.627 (±0.008)*	0.182 (±0.017)*	0.529 (±0.050)*	0.734 (±0.034)*
H22×H29	Control	0.791 (±0.028)	0.476 (±0.231)	0.724 (±0.239)	0.452 (±0.236)
	Drought	0.668 (±0.011)*	0.170 (±0.016)	0.567 (±0.008)	0.805 (±0.037)
Maraton					
H05×AM	Control	0.776 (±0.008)	0.429 (±0.090)	0.733 (±0.063)	0.556 (±0.196)
	Drought	0.601 (±0.051)*	0.141 (±0.002)*	0.574 (±0.000)*	0.843 (±0.022)
AM×H05	Control	0.778 (±0.015)	0.443 (±0.058)	0.737 (±0.041)	0.518 (±0.105)
	Drought	0.518 (±0.082)*	0.158 (±0.026)*	0.612 (±0.044)*	0.816 (±0.108)*

Fv/Fm: parameter characteristic of the quantum efficiency of PSII; ΔF/Fm': actual quantum efficiency; qP: photochemical quenching; qN: non-photochemical quenching; *significant at the P=0.1 level

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STUDIES ON THE EFFECT OF FARMYARD MANURE AND MINERAL FERTILISER ON THE GROWTH OF MAIZE (*Zea mays* L.) IN A LONG-TERM EXPERIMENT. I. USING THE CLASSICAL FORM OF PLANT GROWTH ANALYSIS

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The classical method of growth analysis was applied to compare the effects of farmyard manure (FYM) and mineral fertiliser on the dynamics of growth and growth parameters in maize (*Zea mays* L.) over a three-year period (2005–2007) in a long-term continuous maize experiment set up using the principle of active agent equivalence in 1959. The experiment included two nutrient levels: (i) the NPK active agent equivalent of 35 t ha^{-1} FYM in the form of FYM, FYM + mineral fertiliser or mineral fertiliser alone; (ii) the NPK active agent equivalent of 70 t ha^{-1} FYM in the form of FYM, FYM + mineral fertiliser or mineral fertiliser alone. The aim was to determine the mean and maximum values of the plant growth parameters AGR, ALGR, RGR, NAR and LAR and to compare the effects of FYM and mineral fertiliser on maize growth in various years in a long-term experiment. The effect of the treatments and the year were analysed in terms of the dynamics of total dry matter production, leaf area, absolute growth rate, net assimilation rate and leaf area ratio.

Both the fertiliser treatments and the year had a significant influence on the mean and maximum values of the given growth parameters during the vegetative growth stage. The rate and duration of growth (AGR and ALGR) were lowest in the unfertilised control and highest in treatments given high rates of mineral fertiliser or combined FYM and mineral fertiliser. In all the treatments the significantly lowest values of maximum NAR were observed in 2005, when the weather was average, with higher values in the drier years (2006 and 2007). The maximum values of LAR were significantly the highest in the droughty year of 2007. It could be concluded from the results that the effects of FYM and mineral fertiliser and that of the year on maize growth can be reliably evaluated with the classical method of growth analysis in long-term experiments.

Key words: maize, FYM, mineral fertiliser, growth analysis, classical method, dry matter production, absolute growth rate, net assimilation rate, leaf area ratio

Introduction

Plant growth analysis is a quantitative approach, using only simple basic data, to the description and interpretation of the performance of whole plants growing under natural, semi-natural or controlled conditions (Hunt, 1978).

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Growth analysis was first developed in the 1920s, as described in great detail by Evans (1972). The introduction of growth analysis in Hungary, and its application in crop production, can be attributed to Précsényi (e.g. Précsényi et al., 1976). Such analysis has been underway in Martonvásár since 1956. A detailed, up-to-date account of the principles, methods and application of growth analysis was provided by Berzsenyi (2000). The classical and functional methods of growth analysis, supplemented by agronomic, ecological and physiological measurements, facilitate the scientific, multi-parametric evaluation of the results of crop production experiments.

Many scientists in Hungary have investigated the N responses and optimum N levels of maize hybrids under various edaphic and climatic conditions (e.g. Sárvári, 1995; Futó and Sárvári, 2003). Based on the results of over 40 years of long-term experiments set up on the principle of active agent equivalence, Berzsenyi and Győrffy (1997) reported that the yield was highest in treatments where half or all of the active agent content of FYM was replaced by NPK mineral fertiliser. According to Árendás and Csathó (2002) the efficiency of combined applications of FYM and mineral fertiliser is better than that of FYM alone, and approaches, but does not surpass, that of mineral fertiliser. Soil fertility in continuous maize can be improved without FYM through the regular application of mineral fertiliser. From the agronomic point of view, dry matter accumulation is the most important parameter, so in general this is used as an indicator of growth dynamics (Berzsenyi et al., 2007).

The aim of the present work was to determine and compare the effects of FYM and mineral fertiliser on the growth of maize over several years of a long-term experiment, based on the mean and maximum values of growth parameters for individual plants and the plant canopy.

Materials and methods

Treatments

The long-term, small-plot experiment was set up by Béla Győrffy on partially eroded chernozem soil with forest residues in the experimental nursery of the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár in 1959. The experiment included the following treatments: (1) Control; (2) 35 t ha^{-1} FYM; (3) 17.5 t ha^{-1} FYM + $N_{1/2}P_{1/2}K_{1/2}$ mineral fertiliser; (4) $N_1P_1K_1$ mineral fertiliser; (5) 70 t ha^{-1} FYM; (6) 35 t ha^{-1} FYM + $N_1P_1K_1$ mineral fertiliser; (7) $N_2P_2K_2$ mineral fertiliser (hereafter: Treatments 1–7). The annual active agent quantities applied (kg ha^{-1}) were N 66, P_2O_5 38, K_2O 75 in Treatments 2–4 and N 132, P_2O_5 76, K_2O 150 in Treatments 5–7. The FYM and the P and K fertiliser were applied in autumn every 4 years, most recently in 2006. The 4-year N dose was distributed in equal portions each year. The FAO 380 Martonvásár maize hybrid Norma SC was sown between April 17th and 30th with row and plant distances of 70 × 20 cm. The experiment was laid out in a Latin square design with 7 replications, with a plot size of 80 m^2.

Year effect

The rainfall and temperature data of the three years exhibited substantial differences, compared to each other and to the 30-year mean. The weather was very favourable for maize in 2005 as regards both rainfall and temperature at sowing, thus promoting ideal emergence. In 2006 only half

the usual rainfall quantity was recorded during the sowing period, but the sum for the whole year was around average. The weather in 2007 was extremely hot and dry, with mean temperatures 2°C higher than the long-term mean for every month. The rainfall quantity during the vegetation period (Apr.–Sep.) in the three years was as follows: 526 mm in 2005, 342 mm in 2006, 315 mm in 2007. The mean annual temperature was 9.8°C in 2005, 10.9°C in 2006 and 12.3°C in 2007.

Sampling and measurements

Both the destructive (direct) and indirect methods of growth analysis were applied. Sampling was begun when the maize plants reached the 4-leaf stage of development (22–37 days after sowing) and was continued until physiological maturity. For the destructive analysis three plants were cut at ground level from each replication of each treatment every 14 days. The plants were divided into the following organs: green leaf-blade, stalk + leaf sheath, tassel, ear husk, ear stalk, ear and kernels. Measurements were made on the fresh mass of the plant organs, the leaf area and leaf number, and the dry mass of the separate organs after drying at 105°C for 48–72 h. For the indirect analysis, the leaf area above the ear and the chlorophyll content were recorded for the plant stand.

Classical plant growth analysis

In the present work the effect of the fertiliser treatments was characterised using the classical method of growth analysis, where mean values of the growth parameters are calculated for each sampling period (Evans, 1972). The following parameters were calculated: the absolute growth rate of total dry matter (AGR) and the absolute growth rate of the leaf area (ALGR). For each growth parameter the mean value during the vegetative growth phase, the maximum value and the growth dynamics over the whole growth period were determined. Among the plant growth parameters, AGR is the simplest indicator of plant growth, illustrating the absolute growth rate of dry matter in the whole plant or in individual plant organs.

The modern program published by Hunt et al. (2002) was applied to determine the mean values of the relative growth rate (RGR) and its components: net assimilation rate (NAR) and leaf area ratio (LAR) during the vegetative growth period. The program carries out mathematical calculations and also provides statistical parameters (standard error, confidence limits). RGR is the product of one physiological (NAR) and one morphological (LAR) parameter (RGR = NAR × LAR), where NAR is the index of the productive efficiency of the plants, compared not in terms of total dry matter (as in the case of RGR) but in terms of total leaf area. NAR can be used as an indicator of the growth of both individual plants and of the plant canopy. LAR is the ratio of leaf area to the plant dry mass and reflects the extent of photosynthetic capacity compared with the total biomass.

The data were evaluated using biometrical analysis (Sváb, 1973).

Results and discussion

Effect of FYM and mineral fertiliser on the dry matter production of maize plants and its absolute growth rate

In crop production, growth is generally defined as dry matter accumulation. Differences in the shapes of the growth curves demonstrate the effects of treatments and years on plant growth. Figure 1 illustrates the effect of the application of FYM and mineral fertiliser and that of the year on the dynamics of total dry matter production and absolute growth rate for maize plants, on the basis of measured data.

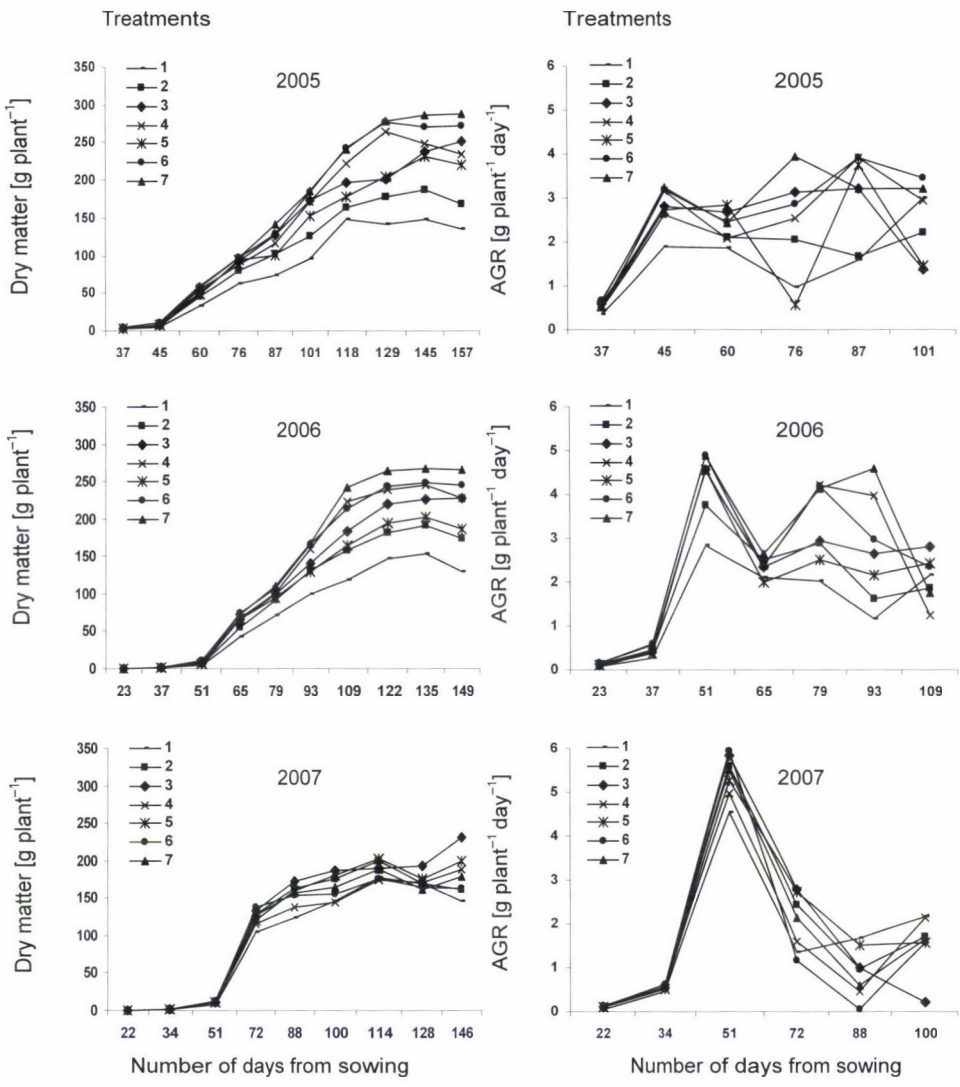

Fig 1. Effect of fertilisation treatments and years (2005–2007) on the dynamics of total dry matter production and absolute growth rate (AGR) of maize plants based on the classical method of growth analysis. Treatments: 1. Control; 2. 35 t ha^{-1} FYM; 3. 17.5 t ha^{-1} FYM + $N_{1/2}P_{1/2}K_{1/2}$; 4. $N_1P_1K_1$; 5. 70 t ha^{-1} FYM; 6. 35 t ha^{-1} FYM + $N_1P_1K_1$; 7. $N_2P_2K_2$

In 2005 and 2006 the various treatment effects were quite distinct: the highest dry matter production (278 and 267 g plant^{-1}) was recorded in Treatment 7 and the lowest (149 and 153 g plant^{-1}) in the control, while intermediate values were observed in Treatments 3, 4 and 5. In 2007 the range of dry matter production values in the individual treatments was much narrower, with the highest values in Treatments 5 and 3 (203 and 230 g plant^{-1}) and the lowest in Treatment 6 and the control (176 and 177 g plant^{-1}).

It can be seen from the slope of the curves that in 2005, thanks to the optimum rainfall supplies, the curve did not reach a maximum at the end of the growth period, indicating that growth continued. In the drier year of 2006 the dry matter production reached a maximum in all the treatments and clearly declined by the end of the growth period. The drought in 2007 resulted in significantly lower maximum dry matter production in all the treatments, and this maximum was reached 2–3 weeks earlier than in 2005 and 2006. The statistical analysis revealed significant differences between the treatments in all three years. Fertilisation-dependent deviations in the dynamics of dry matter production were accurately reflected by the absolute growth rate, the maximum and mean values of which also gave a good characterisation of the year effect. The dynamics of absolute growth rate could be plotted as a bell-shaped curve, i.e. it increased gradually to a maximum (during the leaf development period), after which it declined (Berzsenyi, 1996). The values of AGR could be precisely interpreted up to the flowering period, after which the values exhibited considerable fluctuations.

The typical AGR curve could be observed in the very dry year of 2007, while in 2005 and 2006 the decline after the maximum was reached was only slight and exhibited considerable fluctuation. In 2007 the maximum values of AGR obtained in the various treatments also had a narrower range than in the other two years. In all the fertiliser treatments, the maximum AGR values were lowest in 2005 (1.90–3.24 g plant^{-1} day^{-1}), rising to 2.85–4.90 g plant^{-1} day^{-1} in 2006, with the highest values in 2007 (4.53–5.95 g plant^{-1} day^{-1}).

Effect of FYM and mineral fertiliser on the leaf area of maize plants and its absolute growth rate

The effect of the fertiliser treatments and years was also characterised using the seasonal dynamics of the leaf area and its absolute growth rate (ALGR). The leaf area per plant is genotype-dependent, but is also influenced by environmental and agronomic factors, as are the maximum leaf area per plant and the leaf area duration (LAD) (Berzsenyi and Lap 2006).

The effects of the seven treatments on the seasonal dynamics of the leaf area were clearly distinct in 2005 and 2006, while in 2007 the differences between the treatments were much smaller (Fig. 2). In 2005 the maximum leaf area was smallest in Treatments 1 and 2 (2809 and 2980 cm^2) and highest in Treatments 6 and 7 (4487 and 4581 cm^2). In this year the maximum leaf area was achieved at different dates in each fertiliser treatment. The leaf area stopped growing around 60 days after sowing in Treatments 1 and 2, while it continued to increase up to the 100th day in Treatments 6 and 7. These maximum values were retained for 4–5 weeks in all the treatments, providing an ideal environment for yield formation. In 2006 there was less difference between the maximum leaf area values, with the lowest value in the control (3672 cm^2) and the highest in Treatment 7 (5059 cm^2). The leaf area ceased to increase at approximately the same time in all the treatments, between 78 and 84 days after sowing. The maximum values were only maintained for two weeks in Treatments 1, 2 and 5, but for four weeks in the other treatments. In 2007 the maximum leaf area was highest in Treatment 6 (6017 cm^2) and lowest in Treatment 1 (4778 cm^2). The values recorded in the other treatments had a very

narrow range, from 5479 to 5647 cm². The maximum leaf area was achieved at roughly the same time in all the treatments, between 70 and 75 days after sowing, but due to the rainfall deficiency and atmospheric drought during the summer, the canopy quickly withered, which had a substantial effect on yield formation. Analysis of variance revealed significant differences in leaf area between the various treatments in all three years.

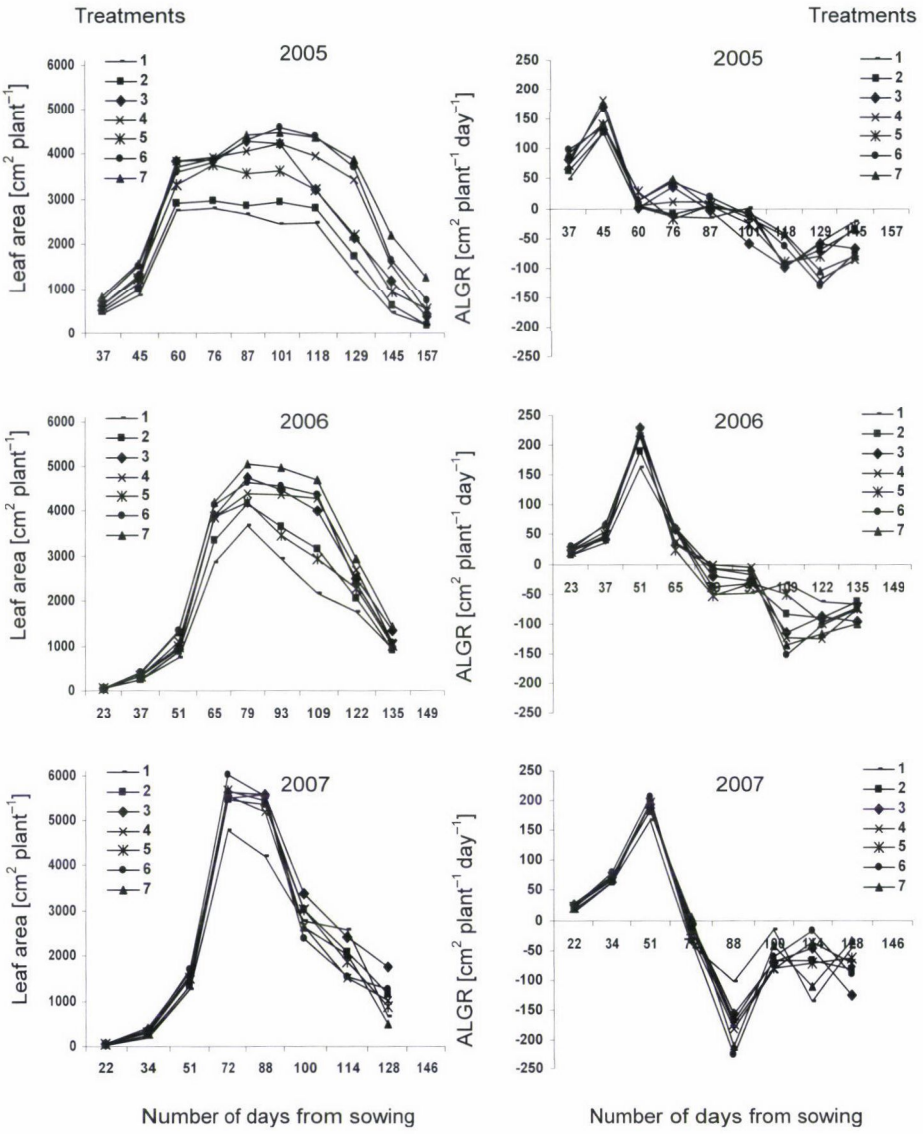

Fig. 2. Effect of fertilisation treatments and years (2005–2007) on the seasonal dynamics of the leaf area and the absolute growth rate of the leaf area (ALGR) of maize plants based on the classical method of growth analysis. For treatments, see Figure 1

The absolute growth rate of the leaf area increased to a maximum (during the leaf development period), then gradually decreased until the end of the growth period (0 value), followed by an intense decline. After reaching a minimum value the rate of withering slowed down. The maximum value of the absolute growth rate was recorded at the same date in all the treatments in 2006 and 2007 (on the 51st day after sowing) and nearly a week earlier in 2005 (on the 45th day). In all the treatments the ALGR maximum values were lowest in 2005 (127–181 cm^2 $plant^{-1}$ day^{-1}), being considerably higher in 2006 (163–229 cm^2 $plant^{-1}$ day^{-1}) and slightly lower again in 2007, with the exception of Treatments 1 and 2 (167–205 cm^2 $plant^{-1}$ day^{-1}). In the driest year, 2007, the curve clearly illustrates the rapid withering of the canopy (i.e. a drastic reduction in the leaf area) during the ear growth stage. In 2005 and 2006 the withering of the canopy was a slower, more uniform process, thus promoting grain filling. The effect of FYM and mineral fertiliser and of the year on the mean and maximum values of the growth parameters AGR and ALGR during the vegetative growth phase is illustrated in Table 1.

Effect of FYM and mineral fertiliser on the net assimilation rate and leaf area ratio of maize plants

The role of the net assimilation rate (NAR) and the leaf area ratio (LAR) in the interpretation of the variability in relative growth rate (RGR) has only partially been clarified (Berzsenyi 1993). Data in the literature suggest that both NAR and LAR may contribute to RGR variability, but LAR seems to be of greater importance (Poorter, 1989). Figure 3 illustrates the effect of fertiliser treatments and the year on the dynamics of NAR and LAR in maize. The seasonal dynamics of NAR revealed a rapid increase during the initial phase of development followed by an equally rapid decrease after the maximum was reached. Fluctuations could then be seen after flowering. In agreement with the literature, significant differences between NAR values were only observed between the years, not between the fertiliser treatments. In all the treatments, the lowest values of maximum NAR were recorded in 2005 (11.81–14.37 g^2 m^{-2} day^{-1}) and the highest in 2006 (18.10–22.05 g^2 m^{-2} day^{-1}), with a slight decrease in all the treatments in 2007 (17.11–18.90 g^2 m^{-2} day^{-1}).

Table 1

Effect of fertilisation treatments on the mean and maximum values of absolute growth rate of total dry matter (AGR, g $plant^{-1}$ day^{-1}) up till flowering and absolute growth rate of the leaf area (ALGR; cm^2 $plant^{-1}$ day^{-1}) during the leaf growth period (Martonvásár, 2005–2007)

Treatment	AGR						ALGR					
	2005		2006		2007		2005		2006		2007	
	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.
1	1.34	1.90	1.33	2.85	1.60	4.53	59.97	127.01	68.28	163.37	50.63	167.28
2	1.64	2.31	1.70	3.77	2.18	5.58	64.90	127.42	77.84	190.65	67.74	196.68
3	1.83	2.80	1.86	4.56	2.32	5.85	85.02	170.13	88.69	228.81	71.59	193.67
4	1.80	3.17	1.88	4.53	1.81	4.97	86.97	181.30	82.32	213.96	65.27	193.22
5	1.87	2.83	1.77	4.57	2.16	5.27	80.74	139.03	78.99	222.17	68.43	196.33
6	1.91	3.20	2.00	4.89	1.97	5.95	83.26	137.66	86.62	217.10	68.96	205.44
7	1.89	3.24	2.06	4.90	2.09	5.50	82.49	142.01	94.12	221.18	72.90	183.91
$LSD_{5\%}$	0.20	0.47	0.26	1.23	0.51	0.91	9.33	22.50	9.31	28.85	7.52	26.30

For treatments, see Figure 1

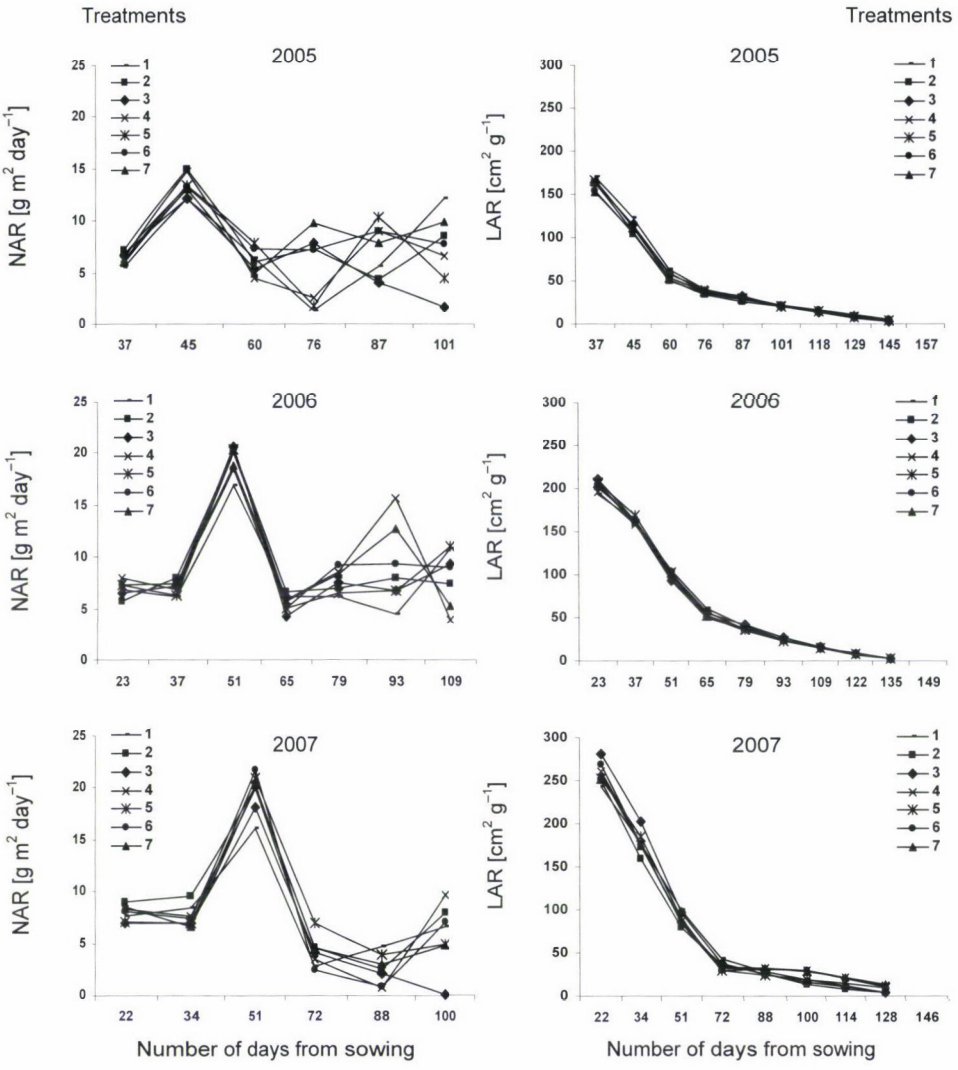

Fig. 3. Effect of fertilisation treatments and years (2005–2007) on the seasonal dynamics of the net assimilation rate (NAR) and the leaf area ratio (LAR) of maize plants based on the classical method of growth analysis. For treatments, see Figure 1

The seasonal dynamics of LAR was characterised by an initial maximum, followed by a gradual decline until the end of the vegetation period. Significant differences in LAR values were observed between both the years and the fertiliser treatments. The maximum LAR values were the lowest in all the treatments in 2005 (154–176 cm^2 g^{-1}), higher in 2006 (205–225 cm^2 g^{-1}) and the highest in all treatments in 2007 (261–315 cm^2 g^{-1}). The effects of FYM, mineral fertiliser and the year on the mean and maximum values of the growth parameters NAR and LAR during the vegetative growth period can be seen in Table 2.

Table 2
Effect of fertilisation treatments on the mean values of relative growth rate (RGR; g g^{-1} day^{-1}) during the vegetative period (Martonvásár, 2005–2007)

Treatment	RGR					
	2005		2006		2007	
	Mean	SE	Mean	SE	Mean	SE
1	0.0676	± 0.0057	0.1032	± 0.0040	0.1079	± 0.0081
2	0.0717	± 0.0073	0.1053	± 0.0029	0.1114	± 0.0083
3	0.0699	± 0.0042	0.1044	± 0.0026	0.1108	± 0.0029
4	0.0720	± 0.0058	0.1072	± 0.0047	0.1071	± 0.0039
5	0.0668	± 0.0067	0.1073	± 0.0054	0.1066	± 0.0034
6	0.0662	± 0.0091	0.1055	± 0.0027	0.1057	± 0.0053
7	0.0658	± 0.0054	0.1070	± 0.0027	0.1063	± 0.0018

For treatments, see Figure 1; SE: standard error

Effect of FYM and mineral fertiliser on the mean and maximum values of growth parameters

In the course of data processing, growth parameters were calculated for each fertilisation treatment at each sampling date. The mean values of the growth parameters then allowed significant treatment effects to be distinguished. The effects of FYM, mineral fertiliser and the year on the mean and maximum values of parameters indicative of the growth of maize plants during the vegetative growth period are summarised in Tables 1–4.

The AGR data calculated from measured data could be interpreted up to the flowering stage and the ALGR data during the leaf growth period, so mean values of these parameters were calculated up to 60–72 days from sowing (Table 1). The maximum values of AGR were the lowest in all treatments in 2005 (1.90–3.24 g $plant^{-1}$ day^{-1}), increasing in 2006 (2.85–4.90 g $plant^{-1}$ day^{-1}) and again in 2007 (4.53–5.95 g $plant^{-1}$ day^{-1}), when the range of values was narrower than in the other two years. Significant differences between the AGR values were found between both years and fertiliser treatments. The year effect was also obvious from the maximum and mean values of the absolute leaf area growth rate (Table 1), with the lowest maximum ALGR values in all the treatments in 2005 (127–181 cm^2 $plant^{-1}$ day^{-1}) and a substantial increase in 2006 (163–229 cm^2 $plant^{-1}$ day^{-1}), while there was a slight decrease in 2007 (167–205 cm^2 $plant^{-1}$ day^{-1}), with the exception of Treatments 1 and 2. Significant differences between the ALGR values were found between both years and fertiliser treatments.

The RGR, NAR and LAR values obtained from measured data can be satisfactorily interpreted during the vegetative growth phase, so the program (Hunt et al., 2002) calculated means for these parameters up to the 79–88th day from sowing. RGR had the lowest value, averaged over the treatments, in 2005 (0.0686 g g^{-1} day^{-1}), while significantly higher values were obtained in 2006 and 2007 (0.1057 and 0.1080 g g^{-1} day^{-1}, respectively). In 2005 and 2007 the lowest values of RGR were recorded in Treatments 5, 6 and 7, with the highest in

Treatments 2 and 4. In 2006 the lowest values were found for Treatments 1 and 3 and the highest for Treatments 4, 5 and 7 (Table 2).

In agreement with the literature, significant differences between the NAR values were only observed between the years, not between the fertiliser treatments (Table 3). Averaged over the treatments, the lowest value of NAR was recorded in 2005 (12.90 g m^{-2} day^{-1}), with an increase in 2006 (17.03 g m^{-2} day^{-1}) and the highest value in 2007 (23.74 g m^{-2} day^{-1}). The maximum NAR values were also the lowest in all fertiliser treatments in 2005 (12.13–14.76 g m^{-2} day^{-1}), rising to 16.88–20.66 g m^{-2} day^{-1} in 2006. In 2007 there was a slight decrease in Treatments 1–4, while in Treatments 5–7 they continued to rise (16.13–21.74 g m^{-2} day^{-1}).

The LAR values also exhibited significant differences between both years and fertiliser treatments (Table 4), with the lowest maximum LAR values in all the fertiliser treatments in 2005 (153.1–170.7 cm^2 g^{-1}), higher values in 2006 (193.4–210.2 cm^2 g^{-1}) and significantly the highest values in all treatments in 2007 (243.0–280.8 cm^2 g^{-1}). Averaged over the treatments, the leaf area ratio was 166.6 cm^2 g^{-1} in 2007, 132.5 cm^2 g^{-1} in 2006 and 102.1 cm^2 g^{-1} in 2005.

Table 3

Effect of fertilisation treatments on the mean and maximum values of net assimilation rate (NAR; g m^{-2} day^{-1}) during the vegetative period (Martonvásár, 2005–2007)

Treatment	NAR)								
	2005			2006			2007		
	Mean	SE	Max.	Mean	SE	Max.	Mean	SE	Max.
1	11.75	± 1.71	12.13	15.52	± 2.42	16.88	22.56	± 3.75	16.13
2	15.06	± 2.98	14.99	17.20	± 1.70	20.24	24.35	± 3.12	20.19
3	12.97	± 1.30	12.16	15.37	± 1.97	20.66	24.04	± 1.31	18.18
4	13.24	± 2.05	14.76	18.39	± 1.15	20.05	22.55	± 4.15	19.88
5	11.68	± 1.36	13.36	18.11	± 1.84	20.40	26.66	± 4.87	20.99
6	12.70	± 2.30	13.32	17.22	± 1.81	18.52	23.70	± 2.68	21.74
7	12.90	± 1.26	13.10	17.39	± 0.90	18.85	22.33	± 1.35	20.24

For treatments, see Figure 1; SE: standard error

Table 4

Effect of fertilisation treatments on the mean and maximum values of leaf area ratio (LAR; cm^2 g^{-1}) during the vegetative period (Martonvásár, 2005–2007)

Treatment	LAR								
	2005			2006			2007		
	Mean	SE	Max.	Mean	SE	Max.	Mean	SE	Max.
1	107.46	± 21.60	170.65	128.26	± 16.56	193.40	156.34	± 70.11	243.00
2	103.28	± 31.07	163.98	128.89	± 11.00	201.74	168.24	± 79.17	250.16
3	103.48	± 17.71	163.09	138.25	± 13.86	210.24	168.85	± 26.88	280.76
4	102.58	± 21.30	164.09	130.01	± 28.17	196.60	169.01	± 25.19	260.66
5	106.70	± 27.19	166.65	133.80	± 36.87	206.82	156.62	± 19.11	253.35
6	96.75	± 33.13	154.06	132.88	± 14.84	204.06	177.04	± 61.17	268.44
7	94.36	± 19.83	153.07	135.51	± 16.01	205.22	170.07	± 21.80	257.26

For treatments, see Figure 1; SE: standard error

Conclusions

The results of analysis of variance demonstrated that various levels of FYM and mineral fertiliser application have a substantial influence on the dynamics of dry matter accumulation and on leaf area growth. The year effect can be clearly characterised by the dynamics of dry matter production and the seasonal dynamics of the leaf area. Different fertiliser treatments had a significant effect on the growth rate of dry matter production (AGR), the absolute growth rate of the leaf area (ALGR), the relative growth rate (RGR), the net assimilation rate (NAR) and the leaf area ratio (LAR). The year effect had a significant influence on the mean and maximum values of the growth parameters during the vegetative growth phase. The growth rate and duration were smallest in the unfertilised treatment and greatest in treatments given high rates of mineral fertiliser or combined FYM and mineral fertiliser. In all the treatments the maximum values of NAR were significantly the lowest in 2005, when the weather was average, while higher values were recorded in the drier years of 2006 and 2007. The maximum values of LAR were significantly the highest in the droughty year of 2007. The results suggest that the classical method of growth analysis can be reliably applied to characterise the effects of various fertiliser treatments on the growth of maize during the vegetative growth period.

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EFFECTS OF INNOVATIVE MICROBIAL MANAGEMENT ON MAIZE (*Zea mays* L.) YIELD IN A LONG-TERM FERTILISATION EXPERIMENT

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Research indicates that there is considerable potential for a successful switch from high chemical use to lower-input, more sustainable farming practices for maize. The overall objective of the MicroMaize project was to field-test the performance of innovative microbiological management strategies. The effect of microbial consortia on maize growth and grain yield was studied in 2008 and 2009 at Martonvásár (Hungary) in a 50-year-old long-term fertilisation experiment. The experiment was set up in a split-plot design with four replications. The main plots were the fertilisation treatments: A: control, without fertilisation ($N_0P_0K_0$), B: $N_{50}P_{24}K_{43}$, C: $N_{100}P_{48}K_{87}$, D: $N_{200}P_{96}K_{174}$, E: $N_{300}P_{144}K_{261}$. Three microbial inoculation treatments were the sub-plots: C0: control, no microbial consortia, C1: *A. lipoferum CRT1 + P. fluorescens Pf153 + G. intraradices JJ 129*, C2: *A. lipoferum CRT1 + P. fluorescens F113 + G. intraradices JJ129*. The results indicated that the microbial consortia had no significant effect on maize growth and yield. In the ecophysiological analyses, the microbial consortia were found to have a significant positive effect on the chlorophyll content and on the protein and nitrogen contents of the grain yield in 2009. The long-term results revealed that the mineral fertilisation treatments and the year had a significant influence on the growth, yield and grain quality parameters of maize. The effect of nutrient supplies and year during the vegetative growth phase of maize could be quantified using the mean values of the absolute growth rate (AGR) for maize shoots and roots and with the nutrient stress index calculated from AGR. Further field investigations on productivity and eco-physiological parameters will be needed to estimate the effect of microbial consortia.

Key words: maize, microbial consortia, shoot/root dry weight, nutrient stress, long-term experiment

Introduction

Maize, a key crop in Europe, responds well to fertilisers and is often grown using high doses of chemical fertilisers, especially nitrogen (Sárvári, 1995; Nagy, 2006; Berzsenyi and Dang, 2008). The likelihood that surface and

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ground waters will be contaminated with chemical fertilisers, particularly nitrate, is high, especially as the sowing density of maize is lower than that of most other crops, resulting in less soil coverage during the early stages of plant development and thus in higher water run-off carrying fertilisers (Németh, 1995; Nagy, 2009; Izsáki, 2010). Research has indicated that there is considerable potential for a successful switch from high chemical fertiliser use to lower-input, more sustainable farming practices for maize (Piotrowski and Rilling, 2008; Frossard et al., 2009). Maize originates from Mexico and is relatively recent in Europe, especially in the more northern regions. There are indications that the microbes associated with maize (including plant-beneficial microbes), which co-evolved with the plant over thousands of years in Central America, have not been entirely exported to Europe along with maize (Estrada et al., 2002). Therefore, unlike other monocotyledonous crops such as wheat or barley, which have been grown in Europe since ancient times, it is likely that maize is still in the process of selecting a maize-adapted microbial community in European soils. This makes maize an ideal candidate crop to focus efforts to reduce chemical fertiliser inputs via the improved management of crop-associated microbial populations (Russo et al., 2005; Gianinazzi and Vosátka, 2004).

The main mineral nutrients required by plants are nitrogen, potassium and phosphorus, and plant nutrition will be suboptimal when the bioavailable soil pool of these nutrients is insufficient (Marschner, 1986). Therefore, the plant is highly dependent on root-associated microbes to enhance nutrient availability, especially in the case of nitrogen and phosphorus (Jansa et al., 2002; Gianinazzi and Vosátka, 2004; Árendás et al., 2004; Sasvári et al., 2009). The main microbial strategies that can be implemented in agriculture to improve plant nutrition and growth are biofertilisation and phytostimulation. These may take place naturally in the rhizosphere, but indigenous biofertilisers/phytostimulators are often present in insufficient numbers to act effectively. The results of field inoculation trials have been highly variable in the past, but in many cases these experiments were not sufficiently integrated with other farming practices, or used only one strain for inoculation. The recent case of Mexico, where more than two million hectares of maize were successfully inoculated with *Azospirillum* PGFR strains (over 40,000 hectares each year), illustrates that it is possible to make inoculation work on a large scale in agriculture (Estrada et al., 2002).

In the MicroMaize EU FP-6 project (2006–2009) a novel strategy was proposed to reduce fertiliser use in maize production. It was based on the exploitation of a major (but so far neglected) component of soil fertility, the soil microbial community, and involved the management of three well-identified soil microbes (the bacteria *Azospirillum* sp. and *Pseudomonas* sp., and the mycorrhizal fungus *Glomus* sp.), integrated with other farming practices in a system-based approach. The MicroMaize project consortium involved eight institutions from six different countries: UMR CNRS Ecologie Microbienne, Université Claude Bernard, Lyon (CNRS, France, coordinator); National

University of Ireland, University College, Cork (UCC, Ireland); Swiss Federal Institute of Technology, Institute of Integrative Biology/Plant Pathology (ETH, Switzerland); Agricultural Research Institute of the Hungarian Academy of Sciences, Crop Production Department (Hungary); Universidad National Autónoma de México (UNAM, Mexico); Institut du Végétal (Arvalis, France); Symbio-M (Czech Republic) and Agrauxine (France). The research methods included molecular biological techniques for the development of microbial consortia, the selection of maize genotypes, greenhouse testing procedures and small-plot field experiments. The main task undertaken by Martonvásár in the project was to implement field trials to assess the performance of the innovative microbial management techniques designed in the project.

Materials and methods

Experimental treatments

The effect of microbial consortia on maize growth and grain yield was studied in 2008 and 2009 in Martonvásár in a long-term fertility experiment established by Győrffy in 1961. The soil of the experimental area was a humus-rich loam of chernozem type with forest residues, poorly supplied with available phosphorus, but well supplied with potassium. Soil analysis was carried out on two of the treatments, the unfertilised control ($N_0P_0K_0$) and the highest NPK dose ($N_{300}P_{144}K_{261}$), in 2008 (Table 1). It can be seen from the table that the $CaCO_3$ content and the pH were higher in the control plots, while the P and K contents and the microelement contents were greater for the high NPK dose.

Table 1
Results of soil analysis on the control ($N_0P_0K_0$) and the highest NPK level ($N_{300}P_{144}K_{261}$) plots in the long-term experiment in 2008

Soil parameters	Unit	Treatments	
		$N_0P_0K_0$	$N_{300}P_{144}K_{261}$
$CaCO_3$	%	1.4	<0.1
Organic C	%	1.8	1.9
Total N (organic + NO_3)	%	0.2	0.2
C/N		9.3	9.2
Organic matter	%	3.0	3.3
pH (H_2O)		8.0	6.4
pH (KCl)		7.3	5.6
Olsen P (P_2O_5)	mg/kg	27.0	143.0
Exchangeable K (K_2O)	mg/kg	328.0	648.0
Exchangeable Mg (MgO)	mg/kg	549.0	494.0
Boron	mg/kg	1.5	1.6
Cu (EDTA)	mg/kg	3.1	4.8
Zn (EDTA)	mg/kg	1.7	1.36
Mn (EDTA)	mg/kg	14.2	39.7

The experiment was set up in a split-plot design, where the main plots were the fertilisation treatments and the sub-plots were the microbial consortia. The randomized block design included five fertilisation treatments (nutrient rates in kg ha^{-1} in subscript): A: control, without fertilisation ($N_0P_0K_0$), B: $N_{50}P_{24}K_{43}$, C: $N_{100}P_{48}K_{87}$, D: $N_{200}P_{96}K_{174}$, E: $N_{300}P_{144}K_{261}$. Three microbial inoculation treatments were established as sub-plots of the fertilisation treatments: C0: control, no microbial consortia, C1: *A. lipoferum CRT1 + P. fluorescens Pf153 + G. intraradices JJ 129*, C2: *A. lipoferum CRT1 + P. fluorescens F113 + G. intraradices JJ129*. The *Azospirillum* and *Pseudomonas* strains were formulated separately on fine peat containing 10^{12} bacteria in 600 g peat, sufficient to inoculate 60,000 seeds. Seed coating was executed by hand before sowing. *Glomus intraradices* was fixed on clay-zeolite substrate, at a concentration sufficient for a dosage of 200 kg ha^{-1}. The clay-zeolite substrate was placed 5 cm to the side of and below the seeds during sowing. Non-inoculated carriers (peat and zeolite) were added to the control treatment (C0).

Sowing was carried out on April 25 in 2008 and April 29 in 2009 using a Wintersteiger Plot Spider seed drill. In both years the inoculant-friendly maize hybrid PR37Y15 was sown at a plant density of 70,000 plants ha^{-1}. Conventional cultivation practices were applied.

The weather conditions were favourable for maize production in 2008, with 483 mm of rainfall during the vegetation period, an average temperature of 18.0°C and cumulated global radiation amounting to 329 MJ m^{-2}. By contrast, in 2009 the weather was very unfavourable for maize production, with a total of only 17 mm rainfall in April and May, compared with almost 100 mm in 2008 (equivalent to the 30-year mean). There was also considerably less rainfall than usual during the vegetation period: 160 mm, which was around half the 30-year mean and a third of that recorded in 2008 (Table 2).

Table 2

Weather conditions during the growing season of maize in Martonvásár in 2008 and 2009

Month of the year	Decade	Precipitation (mm)			Average daily temperature (°C)			Global radiation (MJ m^{-2})		Relative humidity (%)	
		2008	2009	30yrs aver.	2008	2009	30yrs aver.	2008	2009	2008	2009
April	1	4.9	0.1	12	10.1	13.5	10.4	13.7	19.4	68.3	62.9
	2	19.9	2.0	13	12.1	14.0	10.8	15.9	20.1	70.5	65.8
	3	11.4	0	18	13.3	14.4	12.6	18.3	21.7	66.2	53.0
May	1	21.9	0.6	18	14.9	14.8	14.8	19.6	22.5	66.8	59.8
	2	29.6	0.7	16	17.1	17.2	17.0	20.6	21.0	66.6	67.7
	3	11.1	11.3	22	19.1	16.8	17.3	23.2	22.2	65.9	64.6
June	1	102.2	7.4	26	21.0	17.7	19.1	20.4	21.8	70.9	71.0
	2	52.7	12.5	22	18.8	18.9	19.5	20.0	26.3	73.0	63.2
	3	19.1	49.9	25	23.9	18.8	20.6	24.3	14.3	67.2	86.9
July	1	6.0	4.1	18	21.4	20.8	21.0	23.3	23.2	64.0	72.0
	2	61.1	17.6	16	21.6	22.0	22.0	18.8	26.0	70.5	66.3
	3	9.7	1.1	19	21.1	22.6	21.5	16.9	19.1	73.9	67.3
August	1	0.6	23.7	18	22.0	23.0	21.6	20.9	18.6	70.0	68.1
	2	37.6	5.5	15	21.2	21.0	21.0	21.0	20.5	66.8	70.2
	3	6.3	13.6	13	19.8	20.5	19.6	17.9	19.9	64.7	69.2
September	1	21.4	4.1	10	20.7	20.8	18.8	16.4	18.6	66.0	63.9
	2	53.3	17.6	14	12.8	22.0	16.4	8.4	13.7	79.4	72.4
	3	14.2	1.1	17	12.4	22.6	14.6	9.9	14.6	76.1	69.0
Sum/average		482.7	159.5	312	18.0	19.0	17.7	329.4	363.5	69.3	67.4

Field measurements and data analysis

The measurements and observations included the monitoring of maize development and growth (crop phenology, rooting density, flowering date, foliage development and leaf area index), the N status of the whole plant and plant organs, stand homogeneity (density, pest and wind damage), whole shoot biomass at silage harvest, grain yield (analysis of yield components, proportion of barren plants, and protein, starch and oil content of grains), and residual soil nutrient levels after harvest. For the plant analysis, shoot and root mass were measured on four occasions each year, sampling 4–5 plants from each plot. The aboveground plant mass was determined at silage maturity on 15 plants per plot. The leaf area was measured using a portable leaf area meter (LA 300A), the chlorophyll content with a SPAD 502 chlorophyll meter and the leaf photosynthetic rate with a portable LI COR 6400 Photosynthetic System. The nitrogen and protein contents of the whole plants and grain were analysed using the Fiastar 5000 Analyzer and Kjeltec 8400 equipment.

At each sampling the data were first analysed using two-factorial analysis of variance (ANOVA). Data from repeated measurements were evaluated over time with three-factorial ANOVA, considering the measurement date as an additional factor (sub-sub-plot) in the experiment (Gomez and Gomez, 1984). The absolute growth rate (AGR) was calculated for the shoot and root dry weight data according to Hunt (1982). The mean value over the interval t_1 to t_2 is given by: $AGR = (W_2–W_1)/(t_2–t_1)$, where W is the dry weight (g plant^{-1}) of the shoot or root, and t is the time of sampling. Nutrient stress is a quantitative estimate of the intensity of current nutrient deficiency in a plant. The relative shortfall can be expressed as a percentage (Greenwood, 1976): 100 × [(AGR at maximum NPK response) – (AGR at a deficiency)]/(AGR at maximum NPK response).

Results

The effect of mineral fertilisation and microbial consortia on the dry shoot and root mass and on the root/shoot ratio at the four sampling dates in 2008 and 2009 is illustrated in Tables 3 and 4. It is clear from the data that the fertiliser treatments had a significant effect on shoot dry mass at all sampling dates in both years. With the exception of the first sampling date in 2008, the fertiliser treatments also had a significant effect on the root dry mass. The root/shoot ratio exhibited significant effects at all sampling dates in 2008, but only at the first sampling date in 2009. A significant effect of the microbial consortia was only observed for the root/shoot ratio, and only at the 3rd and 4th sampling dates in 2008 and the 3rd sampling date in 2009. The dynamics of the root/shoot ratio was characterised by a continuous reduction over the plant development period, and the ratio was significantly higher in the unfertilised control and at low NPK levels in the early stages of growth (sampling dates 1 and 2 in 2008, and the 1st sampling date in 2009) (Tables 3 and 4). The fertiliser treatments had a significant effect on the biomass and grain yield per plant at silage maturity (sampling date 5) in both years, while the microbial treatments had no significant effect.

Table 3

Effect of fertilisation levels and microbial consortia on the shoot and root dry mass (DM) (g plant^{-1}), the root-shoot ratio (R/S) and grain yield (GY) dry mass (g plant^{-1}) in the long-term experiment in 2008

Treatments	1st sampling (20 May)			2nd sampling (03–04 June)			3rd sampling (15–16 June)			4th sampling (20–22 July)			5th sampling (6–7 Sept)	
	Shoot DM	Root DM	R/S	Shoot DM	Root DM	R/S	Shoot DM	Root DM	R/S	Shoot DM	Root DM	R/S	Shoot DM	GY DM
Fertility levels														
A	0.146b	0.216	1.60a	4.79b	1.06c	0.23a	47.5c	8.55c	0.18b	156.8c	28.6c	0.18b	281.8bc	148.4b
B	0.138b	0.212	1.59a	5.10b	1.19c	0.23a	53.7c	10.41bc	0.19ab	177.4bc	29.3c	0.17b	273.5c	148.3b
C	0.164b	0.222	1.58a	6.51b	1.40bc	0.22a	58.5c	12.27b	0.21a	234.8a	40.3bc	0.17b	329.5ab	175.8a
D	0.200a	0.215	1.17b	9.01a	1.67ab	0.19b	83.8b	17.52a	0.21a	221.7ab	45.1ab	0.20ab	330.7ab	173.8a
E	0.212a	0.219	1.10b	10.32a	2.09a	0.20b	97.2a	17.99a	0.19b	239.8a	56.5a	0.23a	341.1a	179.6a
F-test	***	NS	***	***	***	*	***	***	*	**	**	**	**	*
Microbial consortia														
C0	0.185a	0.221	1.41	7.24	1.49	0.21	66.4	13.75	0.21a	199.3	44.6	0.22a	311.1	162.4
C1	0.163a	0.214	1.42	7.16	1.51	0.22	67.6	13.06	0.19b	213.4	39.3	0.18b	314.1	170.0
C2	0.169ab	0.215	1.39	7.04	1.44	0.21	70.4	13.24	0.19b	205.6	36.0	0.18b	308.9	163.2
F-test	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	*	NS	NS

Means followed by the same letter do not differ significantly at P=0.05 within the same treatment and sampling period; ***, **, *: Significantly different at the P = 0.1%, P = 1% and P = 5% levels, respectively; NS = non-significant; Fertility levels: A: $N_0P_0K_0$, B: $N_{50}P_{24}K_{44}$, C: $N_{100}P_{48}K_{87}$, D: $N_{200}P_{96}K_{174}$, E: $N_{300}P_{144}K_{261}$; Microbial consortia: C0: control, C1: consortium 1, C2: consortium 2

Table 4

Effect of fertilisation levels and microbial consortia on the shoot and root dry mass (DM) (g plant^{-1}), root-shoot ratio (R/S) and grain yield (GY) dry mass (g plant^{-1}) in the long-term experiment in 2009

Treatments	1st sampling (20 May)			2nd sampling (03–04 June)			3rd sampling (15–16 June)			4th sampling (20–22 July)			5th sampling (6–7 Sept)	
	Shoot DM	Root DM	R/S	Shoot DM	Root DM	R/S	Shoot DM	Root DM	R/S	Shoot DM	Root DM	R/S	Shoot DM	GY DM
Fertility levels														
A	0.161b	0.169b	1.09a	1.26b	0.504c	0.42	5.28d	1.79c	0.35	113.0c	26.3b	0.24	198.9b	82.5b
B	0.168b	0.163b	1.03ab	1.42b	0.523c	0.38	6.67d	2.28c	0.33	128.9bc	28.4b	0.22	259.6a	108.9a
C	0.170b	0.168b	1.02abc	1.54ab	0.603bc	0.42	9.41c	3.27b	0.35	144.4b	35.0a	0.25	286.3a	121.4a
D	0.234a	0.191a	0.934bc	1.84a	0.707ab	0.39	12.5b	4.58a	0.37	163.4a	36.4a	0.23	299.8a	125.6a
E	0.224a	0.188a	0.907c	1.92a	0.732a	0.40	14.8a	4.93a	0.33	167.7a	36.8a	0.22	299.9a	130.0a
F-test	**	***	*	*	***	NS	***	***	NS	***	***	NS	**	**
Microbial consortia														
C0	0.190	0.176	1.01	1.56	0.598	0.42	9.51	3.12b	0.33b	147.0	33.4	0.23	260.7	108.1
C1	0.178	0.172	1.01	1.53	0.613	0.42	8.88	3.11b	0.35ab	139.3	32.5	0.24	259.9	108.7
C2	0.207	0.179	0.978	1.70	0.631	0.42	10.79	3.88a	0.37a	144.1	31.9	0.23	286.1	124.3
F-test	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS

Means followed by the same letter do not differ significantly at P=0.05 within the same treatment and sampling period; ***, **, *: Significantly different at the P = 0.1%, P = 1% and P = 5% levels, respectively; NS = non-significant; Fertility levels: A: $N_0P_0K_0$, B: $N_{50}P_{24}K_{44}$, C: $N_{100}P_{48}K_{87}$, D: $N_{200}P_{96}K_{174}$, E: $N_{300}P_{144}K_{261}$; Microbial consortia: C0: control, C1: consortium 1, C2: consortium 2

On the basis of three-factorial ANOVA, significant effects were observed for mineral fertilisation and the sampling date, and for the fertilisation × sampling date interaction. The growth dynamics of dry root and shoot mass at sampling dates 1–4 as a function of fertiliser and microbial treatments is illustrated in Figures 1 and 2 for 2008 and 2009, respectively. It is clear that the shoot and root dry mass increased at different rates in the different treatments. Higher rates of mineral fertiliser (D, E) tended to significantly increase shoot dry mass at all the sampling dates, while the root dry mass was significantly greater at fertiliser levels D and E at all sampling dates in 2009 and at sampling dates 2–4 in 2008. The growth dynamics of the shoot and root also clearly reflect the year effect.

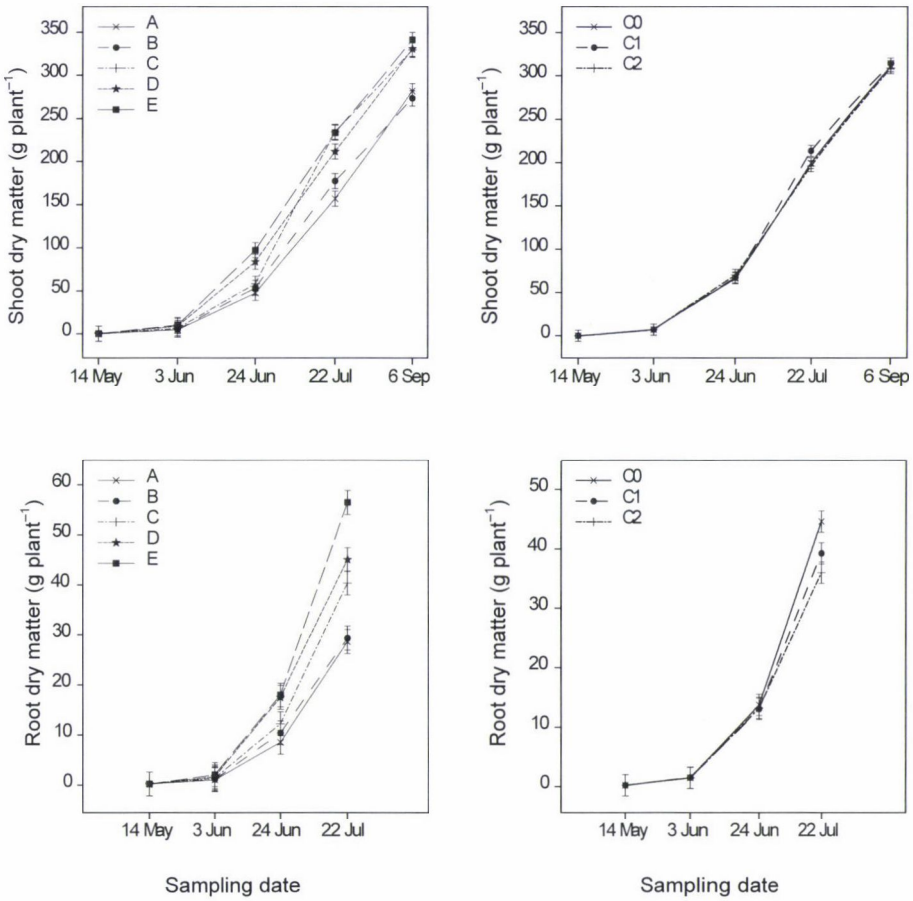

Fig. 1. Effect of fertilisation levels (A–E) and microbial consortia (C0, C1, C2) on the dynamics of shoot and root dry matter accumulation in 2008.
Vertical bars indicate the standard errors of means

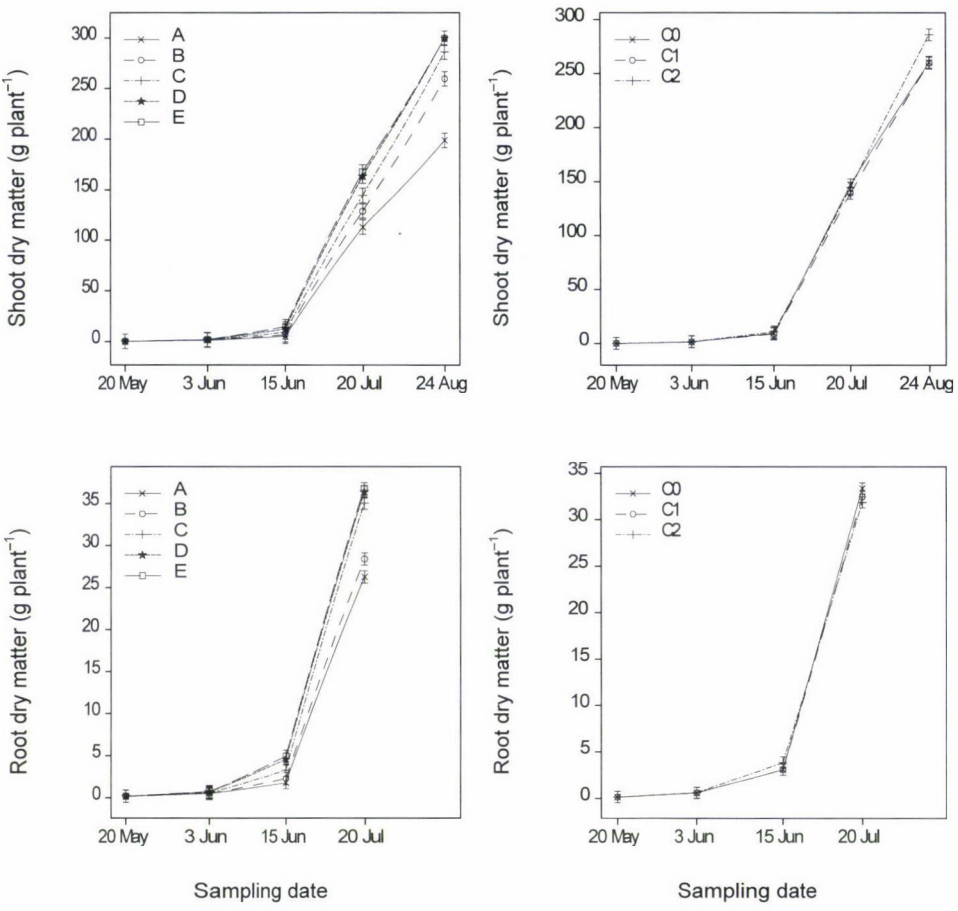

Fig. 2. Effect of fertilisation levels (A–E) and microbial consortia (C0, C1, C2) on the dynamics of shoot and root dry matter accumulation in 2009. Vertical bars indicate the standard errors of means

The root and shoot dry matter accumulation data were used to calculate the mean absolute growth rate (AGR) at each sampling date for each fertilisation treatment. At all the sampling dates the value of AGR was smallest in treatments A and B and greatest in treatments C–E. In 2008 the following AGR values were recorded for maize shoots, averaged over sampling dates 1–4, in the various treatments (g plant^{-1} day^{-1}): A: 2.06, B: 2.33, C: 3.03, D: 2.98, E: 3.25. In 2009, due to the rainfall deficiency, the mean AGR values for maize shoots were significantly smaller over the same period (g plant^{-1} day^{-1}): A: 1.17, B: 1.34, C: 1.54, D: 1.77, E: 1.85. Lower AGR values were recorded for root dry matter accumulation than for the shoots. Averaged over sampling dates 1–4, the following mean AGR values were determined for each fertiliser treatment in

2008 (g plant^{-1} day^{-1}): A: 0.372, B: 0.388, C: 0.526, D: 0.604, E: 0.744, while lower values were obtained over the same period in 2009 (g plant^{-1} day^{-1}): A: 0.277, B: 0.306, C: 0.376, D: 0.423, E: 0.500.

The nutrient stress index was calculated from the AGR values, as suggested by Greenwood (1976). Averaged over sampling dates 1–4, the stress index calculated on the basis of shoot AGR values in treatments A–D had the following values (%) in 2008: A: 36.7, B: 28.3, C: 6.6, D: 8.3, while higher values were recorded in 2009, due to the rainfall deficiency: A: 44.5, B. 35.1, C: 23.2, D: 7.9. The stress factors calculated on the basis of root AGR values were slightly higher, but did not exhibit a year effect. In 2008 the values (%) for each fertiliser treatment were: A: 51.9, B: 47.0, C: 32.1, D: 25.4, and in 2009: A: 43.7, B: 36.8, C: 22.0, D: 4.3.

The effect of mineral fertiliser and microbial consortia on the chlorophyll content (SPAD values), the area of the leaf below the ear (2008) or the leaf area of the whole plant (2009), the grain yield and the grain protein content is illustrated in Figures 3–4. The area of the leaf next to the ear increased significantly in response to mineral fertiliser, exhibiting the following values (cm^2): A: 598, B: 621, C: 671, D: 712, E: 731. A similar significant rise was observed in 2009 for the leaf area of the whole plant (cm^2): A: 4468, B: 4886, C: 5140, D: 5378, E: 5446. The microbial consortia had no significant effect in either year. The SPAD values reflecting the chlorophyll content of the plants rose significantly in response to fertilisation in both years, while microbial consortia only had a significant effect in 2009. The SPAD values for the fertiliser treatments were as follows in 2008: A: 45.2, B: 53.2, C: 56.0, D: 57.9, E: 56.9, and in 2009: A: 46.1, B: 52.6, C: 55.7, D: 58.6, E: 58.7. In 2009 the value recorded for the microbial consortium C2 was significantly greater than the control: C0: 53.4, C1: 54.2, C2: 55.4.

In both years the fertiliser treatments had a significant effect on the grain yield, which rose significantly up to fertiliser level C. The effect of the microbial consortia was not significant in either year. The year effect led to a significantly lower grain yield in 2009, which had poor rainfall supplies. The following grain yields (t ha^{-1}) were recorded at the individual fertiliser levels in the two years: 2008: A: 10.45, B: 12.11, C: 13.71, D: 13.51, E: 12.27; 2009: A: 5.94, B: 7.18, C: 8.3, D: 8.24, E: 8.04. Mineral fertilisation had a significant effect on the grain protein content in both years and the microbial consortia in 2008. The following grain protein contents (%) were found for the different fertiliser treatments in 2008: A: 6.08, B: 7.22, C: 8.10, D: 8.51, E: 8.84, and in 2009: A: 7.33, B: 8.22, C: 8.49, D: 8.91, E: 9.08, while the following values were recorded after treatment with microbial consortia in 2008: C0: 7.60, C1: 7.61, C2: 8.04, and in 2009: C0: 8.3, C1: 8.2, C2: 8.7.

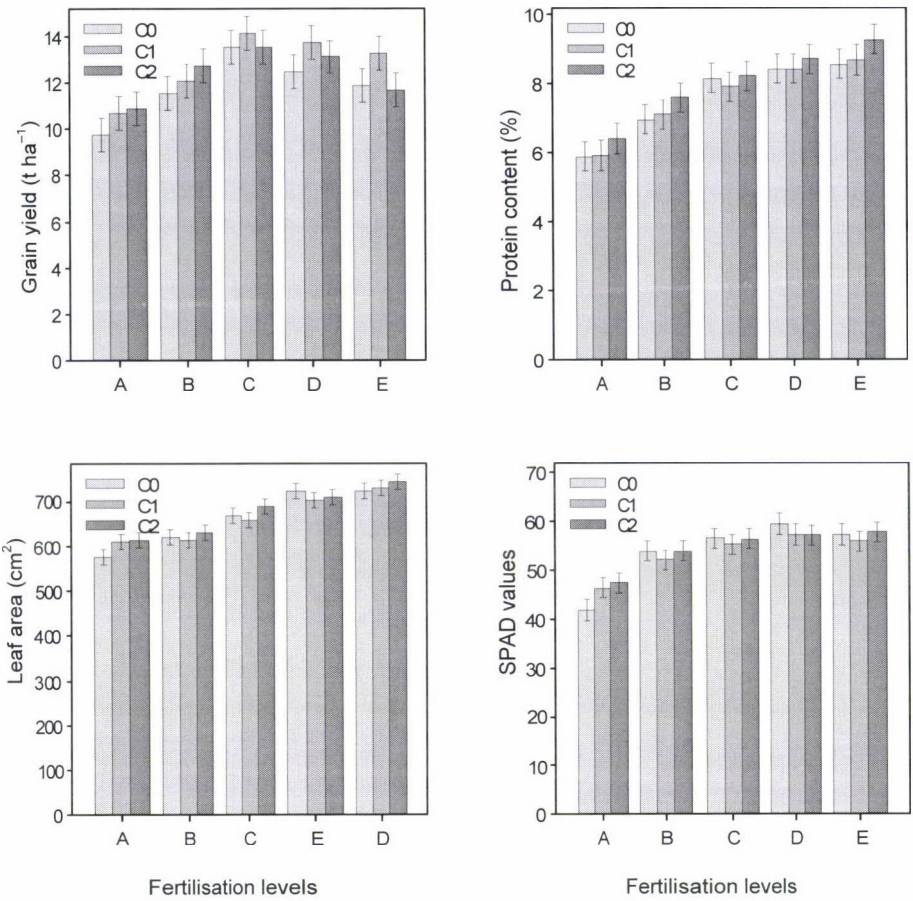

Fig. 3. Effect of fertilisation levels (A–E) and microbial consortia (C0, C1, C2) on the grain yield, grain protein content, area of the leaf below the ear and chlorophyll content (SPAD values) in 2008

Conclusions

As already reported for the results achieved in 2008 (Berzsenyi et al., 2009), no significant effect of microbial consortia was detected in terms of maize growth or yield on the basis of the joint results of field experiments carried out in 2008 and 2009. The Martonvásár findings are in agreement with those reported for trials set up under diverse ecological conditions in other countries involved in the MicroMaize project (France, Italy, Czech Republic, Switzerland, Mexico). The results obtained in Martonvásár may have been influenced by the extremely wet (2008) and dry (2009) weather. Among the eco-physiological analyses, the microbial consortia were found to have a significant

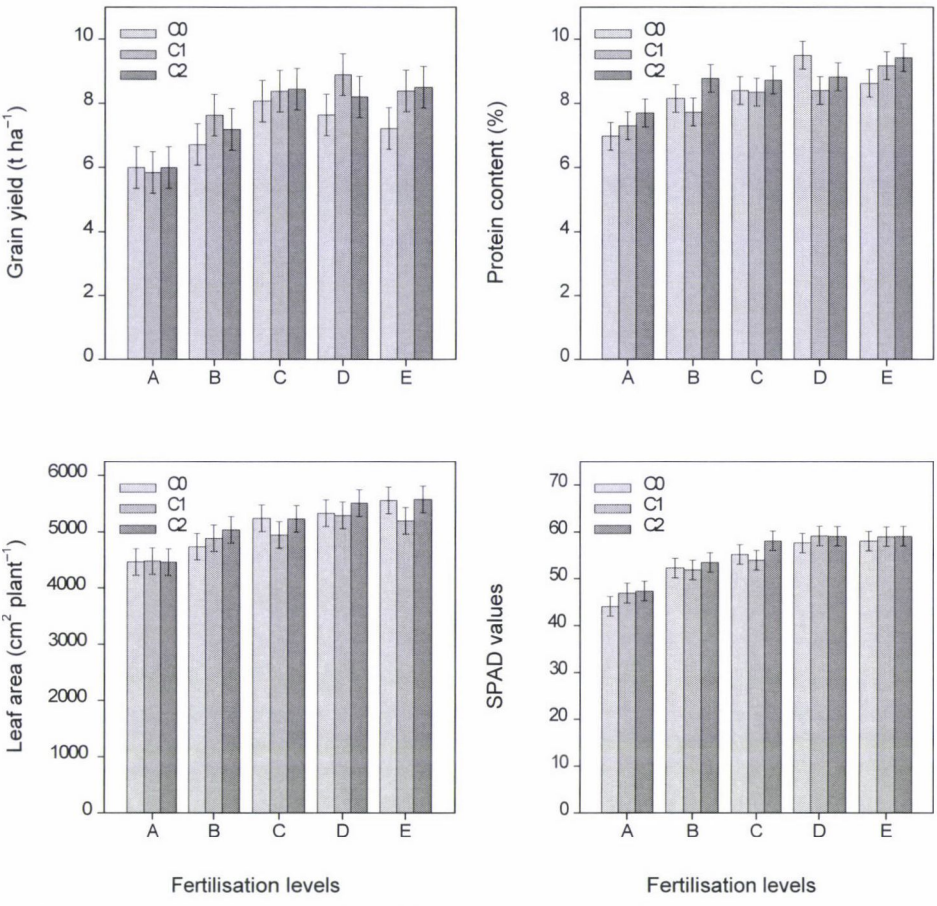

Fig. 4. Effect of fertilisation levels (A-E) and microbial consortia (C0, C1, C2) on grain yield, grain protein content, leaf area per plant and chlorophyll content (SPAD values) in 2009

positive effect on the chlorophyll content and on the protein and nitrogen contents of the grain in 2009. Photosynthetic measurements on individual leaves revealed no significant effect for the microbial consortia. Nevertheless, the results of the long-term experiments gave a good demonstration of the significant effect of fertiliser treatments and the year on the growth, yield and yield quality parameters of maize. Using the mean values of the absolute growth rate (AGR) of the shoots and roots and the nutrient stress index calculated from AGR, it was possible to quantify the effect of nutrient supplies and the year during the vegetative growth period of maize.

The long-term fertilisation experiment provided a unique opportunity to investigate the effect of microbial consortia under different nutrient conditions. In order to obtain a better estimate of the effect of microbial consortia on the productivity and nutrient supplies of maize plants, further field investigations on yields and eco-physiological parameters will be needed in future years.

Acknowledgements

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BREEDING OF CYCLOXYDIM-TOLERANT MAIZE (CTM) HYBRIDS AT THE CEREAL RESEARCH NON-PROFIT CO. LTD.

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The Duo-System technology, which is basically a combination of the Focus Ultra herbicide and cycloxydim-tolerant maize hybrids, is spreading as a tool for weed control in maize crops. The Cereal Research Non-Profit Co. Ltd. commenced the breeding of cycloxydim-tolerant maize (CTM) hybrids based on know-how from BASF. CTM hybrids were created by crossing the CTM inbred lines developed in the initial phase of the programme. The herbicide tolerance of the hybrids was tested in dose rate trials with Focus Ultra in 2008 and 2009. The agronomic value of the novel CTM hybrids was tested in performance trials in 2009. CTM hybrids with high yield potential have been selected as a result of the breeding programme.

Key words: maize, cycloxydim tolerance, performance trial, dose rate trial

Introduction

In Hungary, trials on chemical weed control in maize crops were initiated by Béla Győrffy. Treatments with the herbicides Dikonirt, Atrazine and Simazine were applied, and the data demonstrated that the yield of maize was 5–16% higher on plots with chemical weed management compared to the hoed control (Győrffy et al., 1965).

The active ingredient atrazine provided a convenient solution for weed control in maize crops; nevertheless, its use became an example of how detrimental the long-term application of a single active ingredient may be. The excessive use of atrazine coupled with a permanent monoculture increased the proportion of annual weeds and altered the weed flora (Gyulai and Kocsis, 2009). In the 1970s and 1980s, after the appearance of atrazine-resistant *Amaranthus retroflexus*, efforts were made to find new compounds and modes of action. The increase in weed resistance is a natural process, and this fact should be a warning for the present practice of introducing total herbicides.

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The huge investments made in research and development on herbicides after it was realized that weed resistance could present a risk, resulted in a wide spectrum of active ingredients, formulas, and modes of action.

There are two possible ways of improving chemical weed control. The first is to search for molecules that are not toxic to maize and suppress at least one specific type of weeds, such as mono- or dicotyledonous species. Herbicides, however, were found to possess different levels of crop selectivity, while maize genotypes, especially the parental lines used for F_1 seed production, exhibited different levels of herbicide susceptibility. Based on the results of trials, certain herbicides were banned from seed production in order to avoid damage due to phytotoxicity (Széll et al., 1995).

Safeners were developed to minimize the phytotoxicity of herbicides (Dutka et al., 1984), thus enabling certain efficient, but highly phytotoxic herbicides to become commercially viable.

Currently, computer-assisted programs available online support the scheduling of optimum weed control (Reisinger et al., 2007).

The second possibility is to develop maize genotypes tolerant to the active ingredients in herbicides. The resistance of maize to a herbicide can be achieved either by selecting resistant mutants or by introducing an alien gene into maize (GE).

Maize tolerant to the active ingredient cycloxydim was derived from a population originating from the crossing of two well-known inbred lines via tissue culture.

BASF is the owner of the cycloxydim know-how, which can be used by breeders under contract with BASF. The Duo-System technology, which is a combination of a herbicide with the active ingredient cycloxydim (Focus Ultra) and a cycloxydim-tolerant maize hybrid, is applied to grow CTM hybrids. Focus Ultra is toxic to monocotyledonous seed-grown weeds in the stage of root regeneration, and to *Sorghum halepense*, *Agropyron repens* and *Cynodon dactylon.*

Trials with the Focus Ultra treatment started at the Cereal Research Non-Profit Co. Ltd. in 2000 (Széll et al., 2002), and the programme was extended to include the breeding of CTM hybrids two years later.

Materials and methods

The inheritance of the CTM gene is partially dominant. However, to avoid any risk, only CTM hybrids from the crossing of two parental components with the CTM gene may be introduced into commercial production. Resistant inbred lines are generally developed by backcrossing, but conventional inbreeding was used in the present case to develop resistant maize lines. The breeding process was accelerated in the winter nursery. Herbicide-tolerant stocks were selected after treatment with double the recommended field dose of Focus Ultra. Herbicide-tolerant plants were uninjured, while susceptible ones exhibited anthocyanin discoloration and wilted a few days after the treatment.

The CTM lines were crossed, and the hybrids were tested in performance trials without Focus Ultra treatment, compared with one of the checks in the official trial, and with double the recommended field dose of Focus Ultra. Tolerant hybrids were then tested in a dose rate trial with Focus Ultra.

The performance trial without Focus Ultra treatment was conducted on chernozem soil in a three-replicate Latin block design in Makó. The yields of experimental hybrids were compared to the official check DKC3511. Despite the intensive conditions, only a moderate yield was harvested due to the lack of rainfall in 2009. Conventional weed control was applied.

The CTM nursery and the performance trials with Focus Ultra treatment were conducted at the experimental station of the Cereal Research Non-Profit Co. Ltd. in Újszeged near the river Tisza, on meadow silty soil with medium humus, high phosphorus and potassium content and good water capacity. The experimental design of the performance trials was a Latin block with three replications.

The dose rate trial was set up as an experiment with two factors and four replicates. The treatments for each hybrid involved the following rates of Focus Ultra: untreated control, recommended field dose, and double the recommended field dose. The plants were treated post-emergence at the 6–7-leaf stage. The extent of herbicide injury was determined on the basis of the proportion of plants showing symptoms. The grain yield and grain moisture content were determined for each hybrid. The data were evaluated using analysis of variance.

The trials in Újszeged were irrigated in 2009. There are no irrigation facilities in Makó.

Results

Performance trial without Focus Ultra treatment

Six CTM hybrids bred in Szeged were chosen for testing in the performance trial in Makó in 2009. The control was DKC 3511, the check hybrid in the official trials for the FAO 300 maturity group. The grain yield and grain moisture content at harvest of the hybrids were compared to the control. Two of the six hybrids (CTM1 and CTM4) had yields equal to that of the control, while those of the other four were lower. Only 1–2% difference in grain moisture content at harvest could be observed compared to the control.

Five of the six hybrids (CTM 1–5) were also tested in the weed control trial with Focus Ultra, and three (CTM1, CTM2 and CTM 3) were involved in the Focus Ultra dose rate trial.

Performance trial with double the recommended field dose of Focus Ultra

The yielding ability and herbicide tolerance were tested for twelve CTM hybrids, including CTM1–5, tested in the previous trial, and seven new hybrids.

The hybrid performance was demonstrated by plotting the grain yield (t/ha) against the grain moisture content (%) (Fig. 1). On the graph, the origin represents the mean values for hybrids CTM1, CTM2 and CTM3. When evaluating the data, the axes were taken as being equivalent to the standard level. The most valuable CTM hybrids are those in the upper left domain, characterized by higher grain yield and lower grain moisture content at harvest than the standard. CTM8, CTM 10 and CTM11 can be pinpointed as the best performing hybrids.

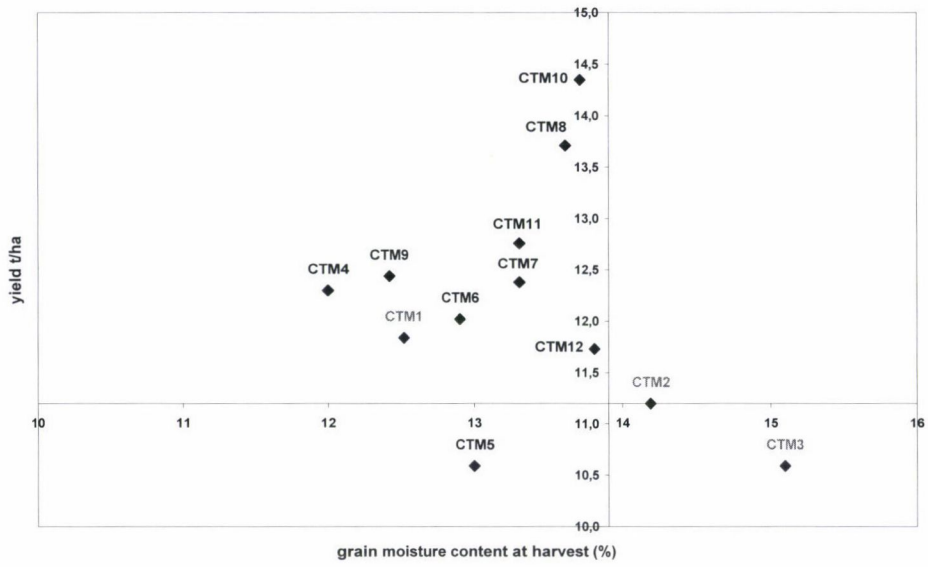

Fig. 1. Performance trial on CTM hybrids treated with the herbicide Focus Ultra

Each of the CTM hybrids proved to be herbicide-tolerant based on the fact that no plants were killed, but some had bleached leaves, indicating a low level of phytotoxicity. The number of plants exhibiting this symptom was recorded for each replication. The data evidenced that bleaching was characteristic of three of the tested hybrids, namely CTM4 (13%), CTM11 (6%) and CTM12 (22%). As plant development progressed, the chlorotic bleaching gradually disappeared.

Testing of tolerant hybrids in the dose rate trial with Focus Ultra

The goal of this trial was to investigate whether Focus Ultra caused any yield reduction in tolerant maize hybrids. The trial was set up as a two-factor experiment in two years, 2008 and 2009. The main factor encompassed two CTM hybrids in 2008, and three, plus the conventionally bred GK Boglár, in 2009. The secondary factor was treatment with the recommended field-rate of Focus Ultra, double the recommended field-dose and an untreated control. The results are summarized in Table 1.

The following conclusions could be drawn from the data.

In general there was no significant difference between the yield of control and treated hybrids. The only exceptions were that the grain yield was significantly higher in one case and significantly lower in another.

Although the difference in yield was not significant, it is interesting to note that the yield increase in 2008 turned into a yield reduction in 2009, as compared to the control. This suggests that the effect of CTM tolerance on hybrid performance exhibits a seasonal effect. The climate was wet and humid in 2009 when Focus Ultra was sprayed, which may have enhanced the uptake of the active ingredient, thus influencing the yield.

Table 1
Grain yield data of CTM hybrids in the trial with Focus Ultra treatment

Experimental years	Code of hybrids	Grain yield on control plots (t/ha)	Difference in grain yield compared to control (t/ha)		
			Recommended dose (3 l/ha)	Double dose (6 l/ha)	$LSD_{5\%}$
2008	CTM2	10.0	+0.2	+0.2	0.4
	CTM3	9.5	+0.8	+0.4	0.6
2009	CTM1	12.0	–0.2	–0.6	0.5
	CTM2	11.2	–0.3	–0.3	0.8
	CTM3	10.9	–0.4	–0.6	0.6
	GK Boglár	11.8	–	–	–

Grain yield varied with the hybrids, but the difference was not significant.

GK Boglár, one of the leading, conventional maize hybrids from Cereal Research Non-Profit Co. Ltd., was included in the trial in order to test the performance of the hybrids compared to a cultivated hybrid and the accuracy of spraying on randomised blocks. GK Boglár plants did not survive on plots treated with Focus Ultra. The data showed that CTM1 yielded on par with GK Boglár, making it competitive with cultivated maize hybrids. CTM2 and CTM3 had the lowest yields in both years. The 2008 and 2009 data confirm the success of the breeding programme.

Discussion

The CTM breeding programme at the Cereal Research Non-Profit Co. Ltd. has resulted in CTM inbreds and experimental hybrids. Hybrid performance was tested in a performance trial, and cycloxydim tolerance in a dose rate trial. The best performing CTM hybrids will be entered for official trials in 2011.

The parental components were bred by conventional breeding and constantly checked for the presence of the CTM tolerance gene. After registration these novel hybrids may be cultivated either with the Duo-System or with conventional technology.

In current practice, the CTM version of a given conventional, commercially released hybrid is often developed. In the official trial, the CTM version is compared to the original normal hybrid, and no difference in DUS results and performance is permitted.

Although this mode of registering the CTM version may appear to be easier, the time pressure induced by the keen competition on the market and the short life-time of hybrids must not be forgotten.

Both solutions have their advantages and disadvantages. They do not contradict, but rather complement each other, and at present both procedures are applied in the breeding programme.

Maize plants not carrying the tolerance gene are killed after cycloxydim treatment. Other plants may be affected by another, more moderate but well-definable impact of cycloxydim, namely leaf bleaching. Smaller or larger bleached patches appear on the leaves, the extent of which may become alarming, but as plant development progresses, the bleaching gradually disappears and the leaves regenerate. This symptom was usually observed in the F_1 or F_2 progenies of hybrids from crosses between CTM and susceptible inbreds. It is thought that the plants exhibiting this symptom carry the tolerance gene, but the enzymatic breakdown of the active ingredient is inhibited.

Each parental component of CTM hybrids intented for introduction in commercial production should carry the tolerance gene, to avoid the risk of farmers being discouraged to grow the novel hybrids.

Sulphonylurea-resistant *Sorghum halepense* has already occurred in the fields, suggesting there may be increasing demand for the Duo-System technology. The task facing researchers is to add valuable germplasm to the novel technology.

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TRADITION, QUALITY AND BIOTECHNOLOGY IN HUNGARIAN SPICE PEPPER (*Capsicum annuum* L.) BREEDING

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Spice pepper production has a history of almost 300 years in the southern part of Hungary. In this study the results of two biotechnological improvements are summarized. Anther and isolated microspore culture techniques were improved to release haploid and doubled haploid (DH) lines for spice pepper breeding. Both the anther and isolated microspore culture methods were successfully used in spice pepper haploid production. Microspore culture- derived structures were analysed to identify their different parts. Green plantlets were regenerated from embryos derived from both anther and microspore cultures. Their doubled haploid analogues were integrated into Hungarian spice pepper hybrid seed breeding programmes. One hybrid, Sláger, was released as a new genotype for spice pepper production in 2008 and two hybrid candidates (Délibáb and Bolero) are now being tested in official trials.

Key words: *Capsicum annuum* L., embryogenesis, histology, microspore culture, ovary co-culture, pepper

Introduction

In southern Hungary, spice pepper growing has a history of several hundred years and in the southern region of the Carpathian Basin a great number of families and medium-sized farms are engaged in spice paprika production. Spice paprika powder (red paprika: sweet and hot) is the most important savoury ingredient in the famous Hungarian goulash and other traditional dishes. Hungary is among the world's five biggest spice pepper producers (Somogyi et al., 2003).

The economic conditions in the 21st century have raised new challenges for spice pepper production. The demand for hybrid seed is increasing on the part of farmers, particularly for spice pepper production in plastic tunnels. Homozygous lines play an important role in breeding programmes. Heterosis

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furnishes new possibilities for the production of spice pepper with higher quality and in larger quantities (Luo et al., 2006). *In vitro* techniques provide a new methodological background for the production of homozygous lines via microspore-derived haploid production (Kasha and Maluszynski, 2003). Methodologically there are two alternatives for *in vitro* haploid production: anther culture or isolated microspore culture. Haploid production is followed by colchicine treatment, which is the last step in DH production.

Anther culture is a well-known method for developing pepper haploids. The use of the procedure to achieve haploid induction was published simultaneously by three different laboratories (George and Narayanaswamy, 1973; Kuo et al., 1973; Wang et al., 1973). Later, Sibi et al. (1979) published a two-step anther culture system, which was further optimized by Dumas de Vaulx et al. (1981). This method was subsequently studied and continuously improved by different laboratories (Mitykó et al., 1995; Dolcet-Sanjuan et al., 1997; Gémes Juhász et al., 1998; 2006; Bárány et al., 2005; Kim et al., 2004) and sporadically applied in different breeding programmes (Thomas et al., 2003; Gémes Juhász et al., 2006; Mitykó and Gémes Juhász, 2006).

Certain aspects of this method, such as genotype dependency, excessive manual work and low efficiency, led to a search for alternative methods for DH plant production. Microspore culture may offer an alternative solution for pepper haploid and DH plant production. Isolated microspores are cultured in liquid medium without somatic tissues, so the embryos and regenerated plants should be derived from haploid cells.

Supena et al. (2006) published the first results of microspore culture-derived haploids in pepper, without giving experimental details, and described androgenesis from shed microspores. Kim et al. (2008) gave a detailed report on the use of the isolated microspore culture method in the hot pepper variety Milyang-jare. Lantos et al. (2009) improved the pepper microspore culture method by using wheat ovaries for alien species ovary co-culture.

The present paper reports the results of anther and isolated microspore cultures of spice pepper genotypes, and gives a short description of the phenomenon of androgenesis in anther and microspore cultures of spice pepper. The *in vitro* haploid induction protocols for spice pepper are based on cereal haploid production methods developed and used in Szeged (Pauk et al., 2003; Lantos et al., 2005) and on internationally published data (Dumas de Vaulx et al., 1981; Supena et al., 2006; Kim et al., 2008).

Materials and methods

Plant material and donor plant growth conditions

The Hungarian and Spanish (from Junta de Extremadura, Servicio de Investigación Agraria, Finca La Orden, Badajoz, Spain) pepper genotypes used in the experiments are essential breeding materials for various pepper research programmes in Hungary. The donor plants were grown in the greenhouse (natural photoperiod, 25–32°C during the day and 15–19°C at night). The seedlings were grown in PVC bags (100 × 180 mm) containing a 1:1 mixture of peat and sandy soil. The donor plants were fed with Volldünger® fertilizer every two weeks.

Collection of donor materials and pretreatment for microspore culture

The donor buds were collected in the optimal developmental stage (late uninucleate and early binucleate stages), sterilized for 20 min in an Erlenmeyer flask containing 50 ml 2% NaOCl solution plus 1 drop of Tween-20, and then rinsed three times with sterile distilled water (Millipore Elix 5). Anthers from sterilized buds were isolated directly into 55 mm diameter glass Petri dishes containing 5 ml 0.3 M mannitol solution and 200 mg l^{-1} cefotaxime (antibiotic). The microspores were pretreated at 32°C in the dark for 7 days.

Anther and microspore culture

For anther culture, the isolated anthers were placed on CP induction medium (Dumas de Vaulx et al., 1981) and kept in the dark at 32°C for 8 days. After heat stress, the cultures were moved to a growth chamber (25°C, 16-hour photoperiod) and the anthers were transferred to R1 regeneration medium after four days.

For isolated microspore culture, the microspore isolation protocol was carried out on the basis of the cereal microspore isolation procedures successfully used earlier (Pauk et al., 2003; Lantos et al., 2005), but with certain modifications (Lantos et al., 2009). The isolated microspores (3×10^4 microspores ml^{-1}) were cultured in 35 mm diameter plastic Petri dishes (Sarstedt Inc., USA, Cat. 83.1800) containing 1.5 ml modified W14 (Ouyang et al., 1989) liquid medium (W14mi) containing 9% maltose, 1000 mg l^{-1} glutamine, 0.5 mg l^{-1} kinetin and 0.5 mg l^{-1} 2,4-D (Ficoll was omitted). 200 mg l^{-1} cefotaxime (antibiotic) was added to each culture. Seven sterile isolated wheat ovaries were added directly to the freshly isolated pepper microspore cultures. The Petri dishes were kept at 28°C in a dark thermostat at high humidity (~80%).

Plantlet regeneration and transfer of plantlets into soil

Microspore-derived embryoids in the bipolar development stage were transferred into 55 mm diameter Petri dishes containing R1 regeneration medium (Dumas de Vaulx et al., 1981). During the regeneration period, the cultures were kept in a culture room at 24°C with a 16/8 hour day/night photoperiod at a light intensity of 100 μmol m^{-2} s^{-1}. When the regenerated plantlets reached the 1–2-leaf stage with roots, they were transferred into glass tubes containing growth regulator-free, half-strength MS medium (Murashige and Skoog, 1962) with 2% sucrose.

The well-rooted plantlets were then transferred into a non-sterilized 1:1 mixture of peat and sandy soil in pots. During the following two weeks, the plantlets were acclimatized in a greenhouse growth cabinet at a relative humidity of 80%. Before flowering, the plants were transferred to an individual isolator box made of wood and cloth. The self-pollination of individuals was assisted with a sterilized brush. The fruits were collected from the microspore-derived plants.

Histological examination

For histological examination of the *in vitro* structures induced in spice paprika microspore cultures, the samples were fixed in a solution of 4% glutaraldehyde buffered to pH 7.2, dehydrated in a graded ethanol series, and then embedded in Historesin (Leica) for light microscopy. A Microm HM 360 microtome was used to cut 6 μm sections. The light microscopy specimens were examined with an Olympus BH-2 epifluorescence microscope.

Results

Androgenesis induction in anther and microspore cultures of spice pepper

Anthers of selected genotypes were isolated from donor buds containing microspores in the ideal developmental stage (Fig. 1a, b). Five to six weeks after isolation, white embryos developed on the regeneration medium, which

regenerated into green plantlets (Fig. 1c, d). Plantlets with two to four leaves were transferred into glass tubes (Fig. 1e). The well-rooted plantlets were acclimatized in the greenhouse (Fig. 1f).

In the isolated microspore culture, viable microspores were separated from the somatic tissues in a white band using modified gradient centrifugation (Fig. 2a). The isolated viable microspores (Fig. 2b) were cultured in W14mi liquid medium with wheat ovaries. The first cell divisions were observed inside the walls of the microspores at the end of the first week. In the presence of wheat ovaries, multicellular colonies emerged from the walls of the microspores in the second week of cultivation (Fig. 2c). The multicellular structures developed intensively (Fig. 2d), and after 5–6 weeks the well-developed embryoids were checked (Fig. 2e). The microspore culture-derived embryos mainly regenerated into green plantlets with leaf rosettes. However, numerous embryos produced normal plantlets (Fig. 2f). The well-rooted plantlets were acclimatized in the greenhouse.

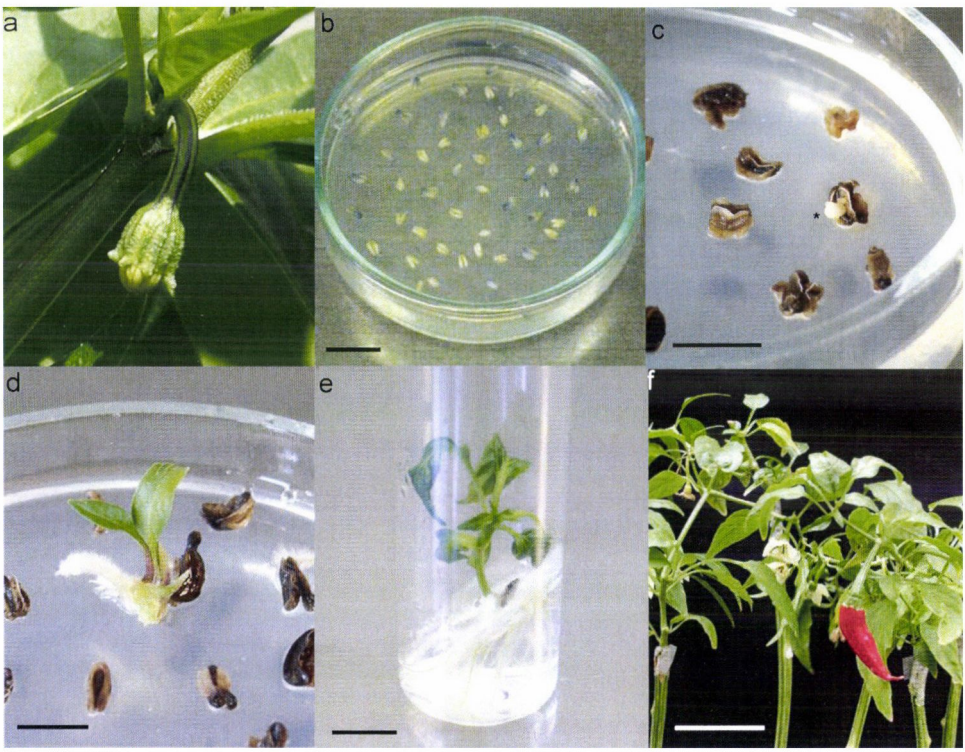

Fig. 1. a A donor bud containing microspores in the optimal stage for the induction of androgenesis, b Isolated anthers on induction medium, c A white embryo derived from anther culture (*), d Green plantlet regenerated from anther culture, e The same plantlet rooted in a test-tube, f Well-rooted plantlets acclimatised to greenhouse conditions. Bars = 1 cm for a, b, c, d and e; 5 cm for f.

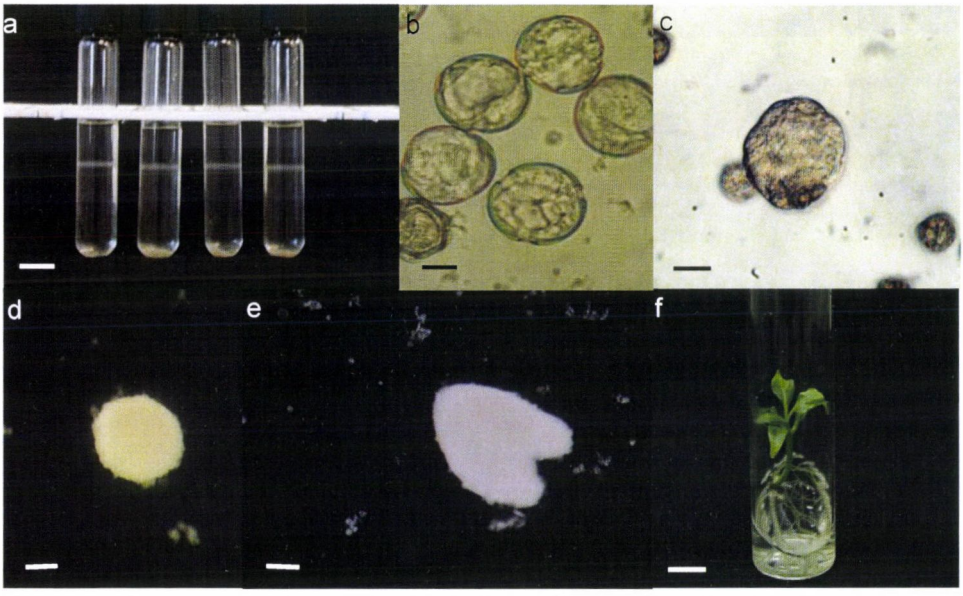

Fig. 2. a Viable microspores form a white band after gradient centrifugation, b Isolated micropores on the first day of culture, c A microspore-derived multicellular structure, d Globular microspore-derived embryos, e A well-developed microspore-derived embryo, f The plantlet was rooted separately in a glass tube. Bars = 1 cm for a and f, 10 μm for b and c, 1 mm for d and e

Histological study of adventitious embryogenesis and the regeneration of spice paprika plantlets

Following the microspore culture experiment, the embryoids obtained were transferred onto R1 regeneration medium. At the end of the first week of the regeneration experiment, well-structured embryoids had emerged and developed. The structure of the microspore-derived embryoids was investigated with an Olympus BH-2 epifluorescence microscope (Fig. 3). The longitudinal section of the slightly elongated embryoids displayed two distinct poles. The root meristem consisted of smaller cells with a lower degree of vacuolization. Vascular differentiation had occurred in the root primordium and the vascular tissues were connected to the central vascular system of the shoot. The opposite pole of the embryoid formed two equally developed primordial cotyledons. A central procambium bundle continued from the hypocotyl into both cotyledons. The central structures were coated with a thick tissue that resembled a cortical layer (Fig. 3). The embryoids were completely covered by hairy epidermis. Epidermal hairs were abundant on the root surface.

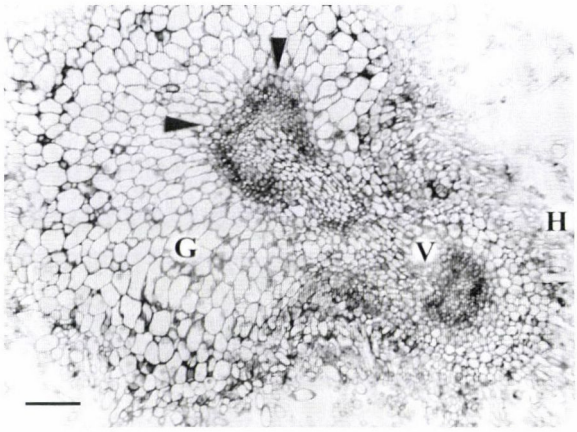

Fig. 3. Longitudinal section of a microspore-derived embryo exhibiting two distinct poles and covered by a hairy epidermis (H). Epidermal hairs are abundant on the root area surface. The vascular tissues (V) of the root primordium are connected to the central vascular system of the shoot. Two primordial cotyledons (arrows) are formed at the opposite pole. The central structures are coated with a thick cortical layer (G). Toluidine blue staining. Bar = 100 μm

Application of DH lines in breeding

The DH lines generated via these methods were integrated into the hybrid programme of the Red Pepper Research and Development Co. Ltd. The homogeneity of the DH lines and the uniformity of hybrids were checked. The homogeneity and agrobotanical traits of the DH lines were checked during seed propagation. No significant variation was found between the parents and their progeny for major phenological and morphological traits.

Discussion

Androgenesis was induced in the Hungarian and Spanish spice pepper genotypes that play a key role in Hungarian spice pepper breeding programmes using anther and microspore culture. The genotype was found to influence the efficiency of the methods. The effect of genotype was investigated in anther culture and shed microspore culture (Mitykó et al., 1995; Gyulai et al., 2000; Ercan et al., 2006; Supena et al., 2006). The genotype influenced the number of embryoids and shoots not only in anther culture but also in isolated microspore culture (Lantos et al., 2009). Plant regeneration from microspore-derived embryos is one of the most critical steps in pepper microspore culture. In the present experiments, the tested genotypes produced microspore culture-derived diploid plants at a rate of 0.2–1.1 plants/Petri dish. Accordingly, further experiments will be required to improve the plant regeneration efficiency and decrease the effect of the genotype in isolated microspore culture.

The longitudinal section of the microspore-derived embryoids displayed two distinct poles. Cell and tissue differentiation were observed on the different parts of the embryoids (root primordium, central vascular system, two primordial cotyledons, procambium and hairy epidermis). These are good indications of the induction of embryogenesis in isolated microspore culture.

Anther culture is already an integral part of pepper breeding (Thomas et al., 2003; Gémes Juhász et al., 2006; Mitykó and Gémes Juhász, 2006), and the new isolated microspore culture will open up new opportunities for breeders. The new anther and microspore culture-derived DH descendants did not exhibit any significant morphological or phenological differences compared to the initial varieties. DH lines play an essential role in Hungarian pepper breeding programmes. The hybrids reached an adequate level of uniformity before they were entered in the national testing system. The hot spice pepper hybrid Sláger was registered in 2008, while the sweet spice pepper hybrids Bolero and Délibáb were still being tested in 2010.

Acknowledgements

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IN SILICO ANALYSIS OF A PUTATIVE *SPIRAL* GENE RELATED TO STRAWBERRY RIPENING

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The aim of this study was to characterize a gene associated with ripening in strawberry, a non-climacteric fruit. Differently expressed transcripts of candidate genes functioning in fruit development and ripening were identified from strawberry (*Fragaria* × *ananassa* Duch.) in four ripening stages using the cDNA-AFLP method. The cDNA fragment designated C11M32M003 was selected from the putative ripening-related genes for further analysis. This transcript accumulated in the green receptacle, and the achene, but gene expression decreased in both tissues in parallel with the progress of ripening (Balogh, 2006). *In silico* analysis revealed that both the cDNA-AFLP fragment (C11M32M003) and the full-length cDNA AY695666 showed over 60% homology at the nucleotide level with two gene groups found in various plant species, including *Arabidopsis thaliana.* One of the candidate groups consisted of *NITRILASE* sequences thought to be related to auxin biosynthesis. As an alternative, a lesser known gene group named *SPIRAL* was suggested. The results of the detailed bioinformatic comparisons presented in this paper prove that the strawberry sequence analysed belongs to the *SPIRAL* gene family.

Key words: *Fragaria* × *ananassa* Duch., non-climacteric ripening, *SPIRAL* and *NITRILASE* genes, *in silico* analysis

Introduction

Based on their ripening behaviour, fruits are classified into two groups: climacteric and non-climacteric. While a large volume of data is available on the genetic background of climacteric fruit ripening (apple, banana, peach), less information can be found in relation to non-climacteric maturation (Giovannoni, 2004). Strawberries, which belong to the non-climacteric group, are not only a valuable horticultural species, but also an important model system for fruit biology studies. In the present experiments, aimed at studying the genetic aspects of non-climacteric ripening, transcripts of candidate genes were

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identified from strawberry (*Fragaria* × *ananassa* Duch.) in four ripening stages using the cDNA-AFLP method (Breyne et al., 2003; Balogh et al., 2005a). The cDNA fragment designated C11M32M003 was selected from the putative ripening-related genes for further analysis. This transcript accumulated in the green receptacle and the achene, but gene expression decreased in both tissues as ripening proceeded (Balogh et al., 2005b). *In silico* analysis revealed that both the cDNA-AFLP fragment (C11M32M003) and the full-length cDNA (AY695666) showed over 60% homology at the nucleotide level with two gene groups found in various plant species, including *Arabidopsis thaliana.* One of the candidate groups consisted of *NITRILASE* sequences thought to be related to auxin biosynthesis (Hillebrand et al., 1998). An alternative, lesser known gene group named *SPIRAL* was also suggested.

Nitrilases function in auxin biosynthesis, hydrolysing nitriles (indole-3-acetonitrile, IAN) to the corresponding carboxylic acid, indole-3-acetic acid (IAA), the most abundant naturally occurring auxin (Hillebrand et al., 1998). The elements of the *SPIRAL* gene family play a role in cell elongation by regulating microtubule organization (Baskin et al., 1994). In *Arabidopsis thaliana* seven members of this gene family are known, of which *Spr*1 and *Spr*2 have been functionally characterized (Nakajima et al., 2004; Furutani et al., 2000; Yao et al., 2008). Recessive mutations of these two genes affect the growth of endodermal and cortical root cells and induce right-handed helical growth of the epidermal cells (Furutani et al., 2000; Yao et al., 2008). This right-handed helical growth can be reverted to the left-handed direction by the drugs influencing microtubule development, e.g. by taxol treatment or by the overexpression of other *SPIRAL* genes in *Arabidopsis* (Bokros et al., 1993; Weerderburg and Seagull, 1988). An understanding of the role of the isolated *Fragaria* gene in fruit ripening will require the functional analysis of the gene and its promoter. As a first step, the aim of this study was the bioinformatic characterization of the selected AY695666 (translated: AAU05601) *Fragaria* × *ananassa* Duch., in order to determine whether it can be grouped into the *NITRILASE* or the *SPIRAL* gene family, in spite of the almost identical sequences of these families.

Materials and methods

Full-length cDNA synthesis

Full-length cDNA of the C11M32M003 transcript was isolated by 5' and 3' RACE reactions (Sambrook and Russell, 2005) using the following primers:

5'-GTTTTGGCCATCTGCACGCATGTA-3'(5'-RACE) and

5'-GAAGAATATGGGTCGTGGAGTCA-3' (3'-RACE) (Balogh, 2006).

RACE reactions were carried out with the Smart RACE kit (BD Biosciences) on mRNA templates. mRNA was isolated from the total RNA of green and ripe fruits (cv. Elsanta) with a Qiagen Oligotex kit (Qiagen, Biomarker).

cDNA cloning and sequencing

For sequencing, cDNA fragments and full-length cDNA were cloned into a pCR2.1 plasmid (Invitrogen) and INVαF' (Invitrogen) *Escherichia coli* cells were transformed with the vector. Plasmid isolation was carried out using a Quantum Prep Plasmid Miniprep Kit (Bio-Rad). The insert was sequenced with an ABI Prism 3100 instrument (Applied Biosystems) in the Agricultral Biotechnological Center (Gödöllő).

Bioinformatic analyses

For the homology search the sequences were first analysed with NCBI BLAST (blastn and blastp algorithms). More detailed sequence comparisons were carried out using the CLC DNA and Protein Workbench, DNAStar Lasergene, MegAlign and Protean softwares. For comparison the following DNA sequences were applied: *Arabidopsis* SPR1 (NM_126416) SP1L1 (NM_001123884), SP1L2 (NM_202381), SP1L3 (NM_111085), SP1L4 (NM_121564), maize *NITRILASE1* (NM_00111211), *Arabidopsis NITRILASE*1, 2, 3, 4 (NM_180680, NM_ 114298, NM_114300, NM_122135).

Results and discussion

The RACE reactions resulted in a 735 bp full cDNA containing a 357 bp protein coding ORF between 96 bp 5' UTR and 282 bp 3' UTR. The ORF codes for a protein consisting of 118 amino acids. NCBI BLAST analysis of the ORF showed higher than 60% homology at the nucleotide level with two gene groups (Fig. 1).

One of the candidate groups consisted of *NITRILASE* sequences thought to be related to auxin biosynthesis (Hillebrand et al., 1998). As an alternative, a lesser known gene group named *SPIRAL* was suggested. The tested *Fragaria* sequence showed 64% and 69% similarity to the *NITRILASE*-associated gene product (AAM64577) and the *sp1l2* (*spiral1-like2*) gene product (AT1G69230), respectively, of *Arabidopsis thaliana*. Neither blastn nor blastp analysis could identify the right *Arabidopsis* homologue of the tested *Fragaria* sequence. So in the next step the translated protein sequences were compared not only with the similar sequences but with all the hypothetical members of the *NITRILASE* and *SPIRAL* gene families (ORF of 5 members of the *SPIRAL* and 4 of the *NITRILASE* gene family). Besides *Arabidopsis* sequences, a maize (*Zea mays*) nitrilase gene sequence was also used for comparison, which displayed 53% homology with the analysed *Fragaria* sequence. Figure 2 demonstrates the number of amino acid substitutions in the translated proteins. The sectors of the SPIRAL and NITRILASE proteins can be unambiguously differentiated, and the tested *Fragaria* sequence clearly belongs to the SPIRAL group. Therefore, *in silico* analysis was continued with comparisons within the *SPIRAL* gene family. The isolated *Fragaria* gene shows high similarity not only to the *Arabidopsis thaliana sp1l2* gene, but also to all other members of the *SPIRAL* gene family, particularly at the obviously very conserved N and C terminal end regions of the proteins, where an almost perfect match can be observed (Fig. 3).

Score	Expected	Identifier	Description
168	1.2e-40	gi|51536941|gb|AAU05601.1|	hypothetical protein [Fragaria x ananassa]
157	2.3e-37	gi|224095996|ref|XP_002311347.1	predicted protein [Populus trichocarpa]
154	2.1e-36	gi|224112102|ref|XP_002316083.1	predicted protein [Populus trichocarpa]
149	1e-34	gi|9797761|gb|AAF98879.1|AC0134	Contains similarity to PIRTA protein from Oryza sativa gb|Z34271 and contains an alpha/beta hydrolase
145	2.2e-34	gi|186478886|ref|NP_001117356.1	SPLL1 (SPIRAL1-LIKE1) [Arabidopsis thaliana]
139	9.4e-32	gi|255542002|ref|XP_002512068.1	SPLL, putative [Ricinus communis]
139	1.2e-31	gi|21592628|gb|AAM64577.1|	putative nitrilase-associated protein [Arabidopsis thaliana]
138	2e-31	gi|255585716|ref|XP_002533540.1	SPLL, putative [Ricinus communis]
137	3e-31	gi|15222255|ref|NP_177083.1|	SPLL2 (SPIRAL1-LIKE2) [Arabidopsis thaliana]
136	6.9e-31	gi|225424637|ref|XP_002285501.1	PREDICTED: hypothetical protein [Vitis vinifera]
133	5.9e-30	gi|147839750|emb|CAN70858.1|	hypothetical protein [Vitis vinifera]
131	1.9e-29	gi|169635145|gb|ACA58349.1|	putative nitrilase-associated protein [Sandersonia aurantiaca]
131	1.9e-29	gi|224141087|ref|XP_002323906.1	predicted protein [Populus trichocarpa]
130	6.2e-29	gi|197312861|gb|ACH63211.1|	nitrilase-associated protein [Rheum australe]
126	8.6e-28	gi|194466083|gb|ACF74272.1|	putative nitrilase-associated protein [Arachis hypogaea]
122	1.2e-26	gi|255547159|ref|XP_002514637.1	SPLL, putative [Ricinus communis]
120	5.3e-26	gi|225455139|ref|XP_002267582.1	PREDICTED: hypothetical protein isoform 2 [Vitis vinifera]
118	1.6e-25	gi|110678955|emb|CAJ38376.1|	putative nitrilase-associated protein [Plantago major]
116	8.9e-25	gi|255628551|gb|ACU14620.1|	unknown [Glycine max]
116	9.2e-25	gi|225423452|ref|XP_002265466.1	PREDICTED: hypothetical protein [Vitis vinifera]
112	1.4e-23	gi|255627397|gb|ACU14043.1|	unknown [Glycine max]

Fig. 1. Results of BLAST analysis on the ORF region of the AY695666 [AAU05601] *Fragaria* sequence. Sequences of high similarity are shaded (red: *SPIRAL*, blue: *NITRILASE*)

		1	2	3	4	5	6	7	8	9	10	11
SP1L1	1		35	41	55	72	70	323	315	324	330	334
SP1L2	2	35		47	60	75	73	324	317	322	330	335
AUU05601	3	41	47		55	75	71	321	313	322	328	335
SPR1	4	55	60	55		70	71	320	313	319	328	334
SP1L3	5	72	75	75	70		34	322	315	322	329	336
SP1L4	6	70	73	71	71	34		320	312	321	324	330
Arabidopsis Nitrilase 1	7	323	324	321	320	322	320		43	57	152	134
Arabidopsis Nitrilase 2	8	315	317	313	313	315	312	43		61	149	131
Arabidopsis Nitrilase 3	9	324	322	322	319	322	321	57	61		152	131
Zea Nitrilase 1	10	330	330	328	328	329	324	152	149	152		112
Arabidopsis Nitrilase 4	11	334	335	335	334	336	330	134	131	131	112	

Fig. 2. Grouping of the members of the *NITRILASE* and *SPIRAL* gene families based on the number of amino acid substitutions. Intensity of red and blue colours shows the extent of alterations

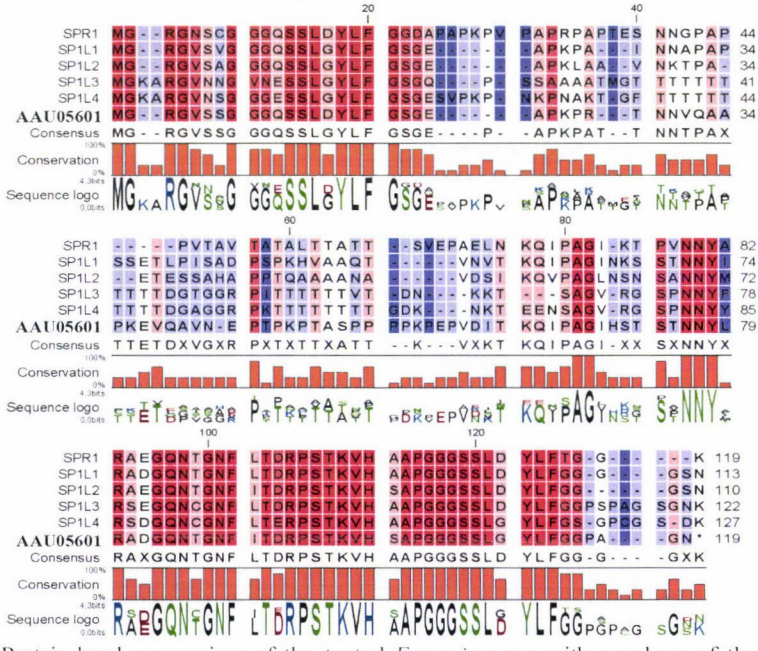

Fig. 3. Protein level comparison of the tested *Fragaria* gene with members of the *Arabidopsis thaliana SPIRAL* gene family using the ClustalW method

These conserved sections, identical in all members of the *SPIRAL* gene family, can also be found in the tested *Fragaria* sequence. It is also striking that the strawberry protein sequence is more similar to the *Arabidopsis* gene *sp112* than the other *SPIRAL* genes of *Arabidopsis* to each other. The members of the *NITRILASE* gene family of *Arabidopsis* showed greater similarity to the maize *NITRILASE* gene than the examined *Fragaria* sequence (Fig. 2).

Cluster analysis, resulting in a phylogenetic tree including the members of the *SPIRAL* and *NITRILASE* gene families of *Arabidopsis thaliana* and the maize *NITRILASE1* gene, placed the strawberry AY695666 [AAU05601] sequence unambiguously in the *SPIRAL* gene family (Fig. 4).

The *NITRILASE* genes are clearly separated from the *SPIRAL* group, suggesting that the isolated cDNA can be regarded as a *SPIRAL* gene of strawberry origin.

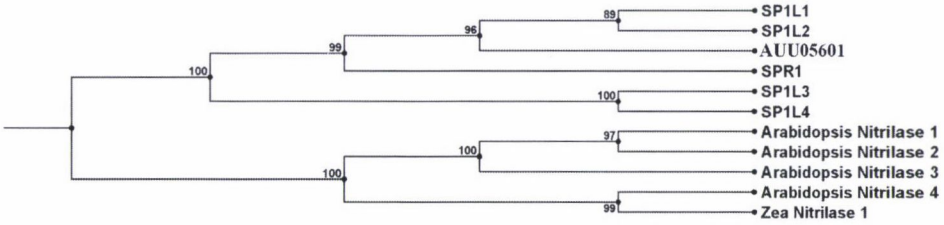

Fig. 4. Phylogenetic clustering of *NITRILASE* and *SPIRAL* genes

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BREEDING *Rosa* TAXA NATIVE TO THE CARPATHIAN BASIN FOR FRUIT PURPOSES – FRUIT QUALITY

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The aim of dogrose breeding for fruit purposes is to select genotypes suitable for cultivation and to produce new genotypes by crossing. Physical and chemical analyses, prospective genotypes have been developed from *R. inodora*, *R. corymbifera*, *R. rubiginosa* and *R. canina* varieties.

In the course of the investigations, the highest vitamin C content was found in the hips of *R. inodora* and *R. rubiginosa*. The glucose and fructose contents ranged from 9.57–13.36 g/100 g, averaged over several years. The amounts of these two carbohydrates were equal, or in some taxa (e.g. *R. corymbifera, R. canina* Sz3) the fructose content was higher.

The glucose, fructose and vitamin C contents changed at different rates in each taxon during ripening. The results showed that the fructose content reached its peak a week earlier than the glucose content. The vitamin C content of morphological varieties of *R. canina* did not change substantially during ripening.

Key words: rosehip, vitamin C, glucose, fructose, ripening

Introduction

The utilization of roses for fruit purposes and the production of cultivars began in the 18–19th centuries in numerous countries of Europe and Asia. Cultivars suitable for today's cultivation requirements have been produced in the last three or four decades. In the main rosehip breeding countries (Sweden, Bulgaria, Slovakia, Poland, Germany, Turkey and countries of the former Soviet Union) taxa belonging to the sections *Caninae* (*R. canina* L., *R. villosa* L., *R. rubiginosa* L., *R. dumalis* Bechst. em. Bouleng), *Cinnamoneae* (*R. majalis* J. Herrm. em. MANSF., *R. rugosa* Thunb., *R. pendulina* L.) and *Synstylae* (*R. multiflora* Thunb.) are bred for fruit purposes (Anonymous, 1999; Koch and Grope, 1993; Uggla and Nybom, 1998; Uggla and Martinsson, 2005; Ercişli, 2005; www.vuood.sk; Wiśniewska-Grzeszkiewicz, 1999).

The breeding of dogrose cultivars suitable for the ecological conditions of Hungary has been underway in the Department of Pomology of Corvinus University of Budapest in cooperation with the Department of Botany since the mid-1990s, based on the extremely rich *Rosa* genetic material found in Hungary (Facsar, 1993; 2005). Besides the 20 rose species belonging to sect. *Caninae* that are native to Hungary, the naturalized species *R. rugosa* Thunb. (sect. *Cinnamomeae*), *R. spinosissima* L. (sect. *Pimpinellifoliae*) and *R. blanda* Alt. (sect. *Cinnamomeae*) and the cultivated relic *R. villosa* L. subsp. *sancti-andreae* (Deg. et Trtm. ex Jáv.) Soó were also investigated (Kovács et al., 2004; 2005; Tóth et al., 2005). The selection of candidate cultivars has been started from the following species: *R. inodora* Fr. (syn. *R. elliptica* Tausch), *R. zalana* Wiesb. and *R. corymbifera* Borkh. The species *R. rubiginosa* L. and morphological varieties and microspecies of *R. canina* L. have also been included in breeding.

The present paper summarizes the main physical characteristics of the pseudofruits (hereinafter: fruits) of major species based on the results of more than 10 years. Among the chemical parameters, the contents of two main components, vitamin C and carbohydrate (sugars), were measured. Earlier publications reported 130 mg/100 g to 6694 mg/100 g vitamin C content in the hips of certain *Rosa* taxa (Uggla et al., 2005; Koch and Grope, 1993; Porpáczy, 2003; Ercişli, 2005; 2007). Relatively few authors have discussed the carbohydrate content of rose hips, reporting values of 10–20 g/100 g carbohydrate, the majority of which is glucose and fructose, while sucrose is only present in insignificant quantities (Brodmann, 1993; Koch and Grope, 1993; Kovács et al., 2004; Uggla, 2004).

Research is currently underway to track changes in chemical content during fruit ripening and to determine optimal harvest time. Changes in the size, hardness and colour of the hips can be used for the visual determination of optimal harvest time (Uggla et al., 2005; Müller, 1997; Brodmann, 1993; Mamadrizohonov et al., 1994). The physical and chemical changes taking place during the ripening of rose hips were discussed in more detail by Uggla (2004), Uggla et al. (2005) and Müller (1997). Data show that the vitamin C, glucose and fructose contents of the fruits vary from species to species and from cultivar to cultivar.

Materials and methods

The rose species included in the investigations originated from the live *Rosa* collection of the Soroksár Botanical Garden of Corvinus University, the Buda Arboretum, the Experimental and Research Station of the Faculty of Horticultural Science in Szigetcsép, and from the hills around Szentendre. Between 1996 and 2009 the fruits of a total of 19 species and 3 varieties with microspecies value were investigated. Several taxa seemed to be promising (*R. corymbifera*, *R. rubiginosa*, *R. inodora*, *R. canina*), and those with the most favourable parameters were selected for further studies. The following points were considered during selection:

- large fruit size, abscission of calyces during ripening, low achene content,
- good acid and sugar content, high vitamin C content,
- simultaneous ripening, retention of quality for a lengthy period on the bush,
- medium strong growth, upright bush habit, good regeneration ability.

When sampling, an effort was made to pick rosehips from all parts of the bush, without concentrating on samples characteristic of the given species. Fifty to eighty ripe fruits were investigated each year for each taxon, and the picking time was chosen to coincide with the ripening time of the given taxon. The diameter, length, hip mass, flesh weight, pedicel length and number of achenes of freshly picked whole rosehips were measured within a week. In order to evaluate the chemical changes taking place within the rosehips, several harvest times were chosen, starting when the fruit began to colour. The total weight and flesh weight of 30–40 hips were recorded at each sampling date.

The chemical content was analysed in the Central Laboratory of the Faculty of Food Science (1997–2000) and in the Chemical Content and HPLC Laboratory of the Department of Pomology. At the beginning, dried whole and half rosehips were used for analysis, but since 2007 lyophilized rosehip flesh samples have been used. Between 1997 and 2000 the vitamin C content of the fruits was determined using the α,α'-dipyridyl photometry method, while since 2007 ascorbic acid has been measured by HPLC. The carbohydrate content was detected by HPLC in the laboratories of Corvinus University of Budapest. In 2007, in cooperation with the Laboratory of Food Analytics of the Central Food Research Institute (Boehringer Mannheim), the UV method was used (measurement of D-glucose and D-fructose concentration).

Results

The best results for fruit size and weight, averaged over several years, were obtained for *R. canina* Sz2 (2 g), the mixed prickle variety of *R. canina* (1.34 g) and *R. corymbifera* Sz3 (1.68 g) (Table 1). The fruit flesh ratio of the taxa varied between 60 and 75%, while the achene content was moderate (17–23/fruit). In *R. corymbifera* and *R. canina* the calyces are abscised during ripening, while the calyces of *R. rubiginosa* persist, but are easy to remove.

The vitamin C, glucose and fructose contents of the taxa are also illustrated in Table 1, in terms of raw fruit flesh. The largest quantity of vitamin C was measured in the fruits of *R. inodora* (485.1 mg/100 g) and *R. rubiginosa* (599 and 431 mg/100 g). The glucose and fructose contents varied between 9.57 and 13.36 g/100 g. The lowest glucose and fructose contents were found in the fruits of *R. rubiginosa* (9.57 and 10.87 g/100 g) and *R. canina* Sz2 (10.79 g/100 g) and the highest in those of *R. corymbifera* Sz3 (13.36 g/100 g). Sucrose could only be detected in quantities of 0.1 g/100 g, if at all. The glucose–fructose ratio was around 1:1 for all the taxa, with the exception of *R. corymbifera* and *R. canina* Sz2, where this ratio was 1:2.

The chemical content of the rosehips was found to change during ripening. The changes observed for vitamin C, glucose and fructose, which differed for each species, are illustrated using the values recorded in 2007.

Table 1
Main pomological and chemical characteristics of the fruits of *Rosa* taxa (averaged over the years 1996–2008)

Taxon	Origin	1	2	3	4	5	6	7	8	9	10
R. inodora 2	SBG	15.99	12.59	1.20	1.13	70.26	17.20	485,1	5.63	6.2	0.909
R. corymbifera	SBG	15.02	11.74	1.27	1.04	68.96	19.84	294,7	5.2	6.89	0.754
R. corymbifera Sz3	Sz	17.72	13.42	1.32	1.68	57.79	22.21	420,5	5.16	8.2	0.630
R. rubiginosa 1	SBG	15.81	10.85	1.46	0.90	59.78	22.95	599.0	4.68	4.89	0.956
R. rubiginosa 2	SBG	17.48	11.85	1.48	1.14	61.72	26.06	431,0	5.67	5.2	1.090
R. canina Sz2	Sz	22.30	14.09	1.59	2.07	65.49	27.36	292,6	3.83	6.97	0.549
R. canina^{+}	SBG	21.01	10.01	2.10	1.08	75.27	21.93	285,5	5.86	6.42	0.912
R. canina^{++}	SBG	17.15	11.62	1.48	1.34	69.41	19.02	217,2	5.2	6.3	0.829

1: Length (mm); 2: Diameter (mm); 3: Shape index; 4: Mass (g); 5: Fruit flesh ratio (%); 6: Achenes per fruit; 7: Vitamin C (mg/100 g); 8: Glucose (g/100 g); 9: Fructose (g/100 g); 10: Glucose–fructose ratio; SBG – Soroksár Botanical Garden; Sz – Szigetcsép; Pomological characteristics refer to fresh whole fruit, and chemical contents to raw fruit flesh; $^{+}$: pendant bush variety; $^{++}$: mixed prickle variety

The highest vitamin C content was found, almost up to the end of ripening, in the fruits of *R. rubiginosa* 2 and *R. inodora* 2 (Fig. 1). The extremely low content (~50–70 mg/100 g) measured in the morphological varieties of *R. canina* did not differ greatly during ripening. The vitamin C content of *R. inodora* fruits reached a maximum in mid-October. The changes taking place during ripening followed a saturation curve. The fruits of *R. corymbifera* and *R. rubiginosa* reached their first vitamin C peak in early October, but a further rise was observed up to the last harvest date.

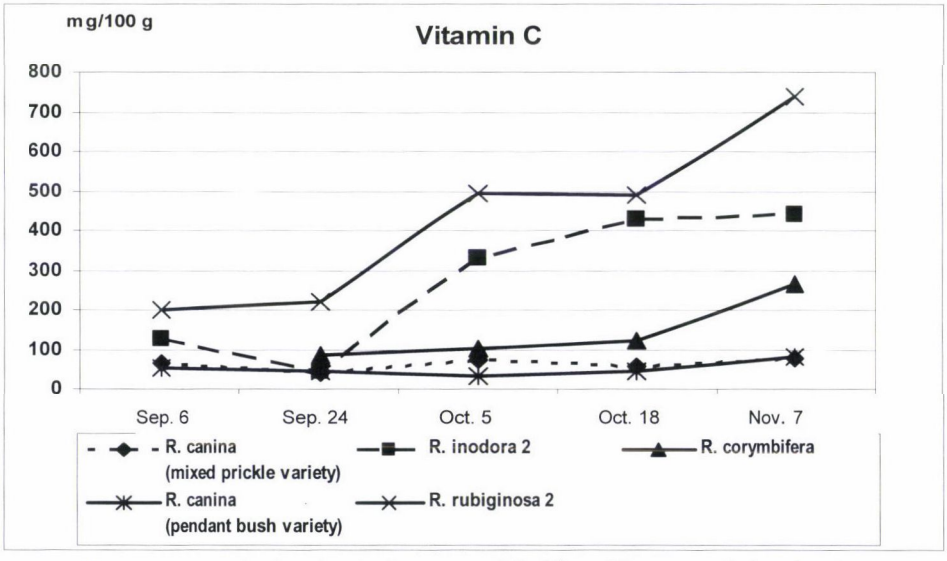

Fig. 1. Changes in the vitamin C content of the hips of *Rosa* taxa during ripening (Soroksár Botanical Garden, 2007; data refer to raw rosehip flesh)

During ripening rises in the glucose and fructose contents were observed in all the taxa evaluated. However, the fructose content (Fig. 2) peaked a week earlier than that of glucose (Fig. 3). The glucose and fructose contents changed at similar rates during ripening in all the species tested. In the case of *R. rubiginosa* the changes followed a saturation curve, while in *R. inodora, R. corymbifera* and the mixed prickle variety of *R. canina,* after reaching a maximum, the values tended to decrease, so the changes followed a Gauss curve. In the pendant bush variety of *R. canina* the glucose and fructose contents first peaked in early October, then started to increase again in early November.

Discussion

In breeding rosehips for fruit purposes the fruit size and fruit flesh ratio are important aspects, which are determined by the parents used in breeding. The fruit parameters of *R. canina* Sz2 and the pendant bush variety approached that of selected cultivars of *R. canina* (e.g. Plovdiv1, Sylwana) with a fruit weight of 1.6–2 g and a fruit flesh ratio of 60–70% (Brodmann, 1993; Müller, 1997; Milewski, 1974; Porpáczy, 1993). The fruit weight (1.5–1.9 g) and fruit flesh ratio (64.3–71.7%) of the *Rosa rubiginosa* types used for fruit purpose breeding in Sweden (Uggla, 2004) are similar (Table 1).

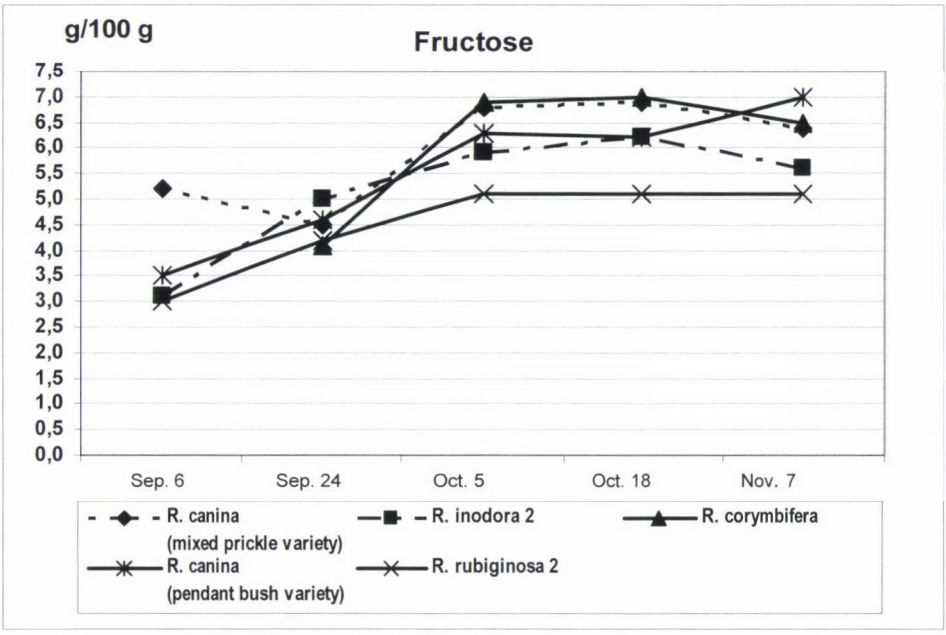

Fig. 2. Changes in the fructose content in the hips of *Rosa* taxa during ripening (Soroksár Botanical Garden, 2007; data refer to raw rosehip flesh)

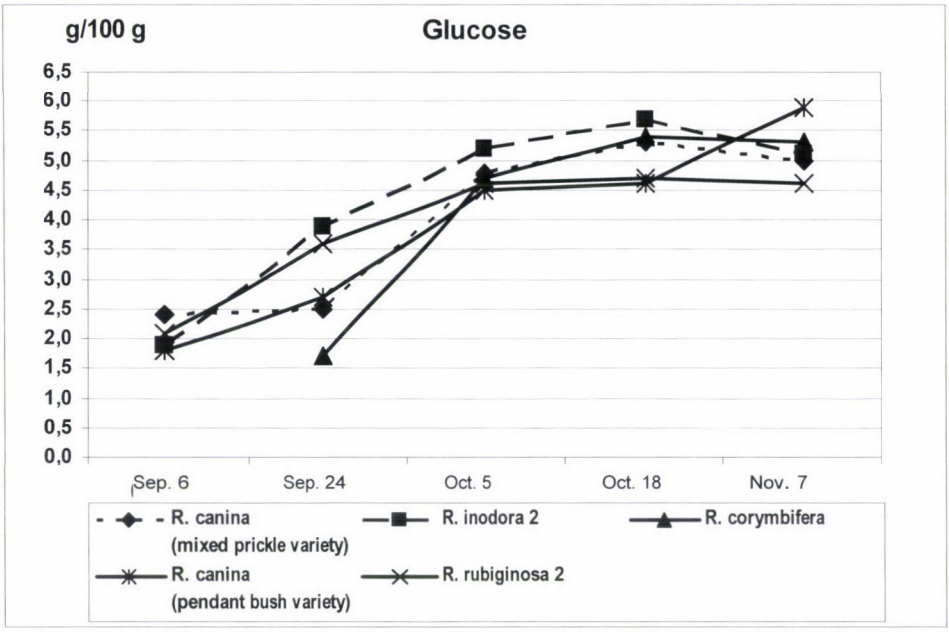

Fig. 3. Changes in the glucose content in the hips of *Rosa* taxa during ripening (Soroksár Botanical Garden, 2007; data refer to raw rosehip flesh)

According to the investigations of Keipert (1981) the fruit of wild-growing roses contain 200–800 mg/100 g vitamin C. The vitamin C content of the rose species analysed in the present experiments also varied between these limits. The species with the highest vitamin C content included *R. inodora* (485.1 mg/100 g) and *R. rubiginosa* (599 and 431 mg/100 g, Table 1). Koch and Grope (1993) and Ercişli (2007) reported significantly higher vitamin C contents in the fruits of *R. canina*. In evaluating reports on the vitamin C content, however, it should not be forgotten that the ascorbic acid content is influenced by a great number of factors, for instance the species, the cultivar, the location, the ripening stage of the fruits, the climatic conditions predominant during fruit development, and how the fruits were stored and processed.

The glucose and fructose contents of the species investigated here amounted to 9.57–13.86 g/100 g, averaged over several years (Table 1), which does not differ substantially from the data published by Brodmann (1993), Koch and Grope (1993) and Uggla (2004). The latter author suggested that the glucose and fructose contents might vary with the location, and found that the amounts of these two carbohydrates were also influenced by the year. In Sweden glucose was predominant in the hips of *R. dumalis* and *R. rubiginosa*, while in Hungary the amounts of the two carbohydrates were the same in *R. rubiginosa*. Among the species investigated, the fructose content was highest in the fruits of *R. corymbifera* and *R. canina* Sz2. The glucose–fructose ratio appears to be correlated with the taxonomical position of the species. In sect. *Rubiginosae* (*R.*

rubiginosa, R. inodora) the glucose and fructose contents were nearly the same, while in sect *Eucaninae* (*R. canina, R. corymbifera*) the fructose content was higher.

According to Müller (1997) and Mamadrizohonov et al. (1994) the ideal time for harvesting rosehips is when the vitamin C content of the fruits reaches a maximum. This varies with the species and the cultivar. The vitamin C content gradually decreases after the optimal harvesting time, and may be as much as 20% lower a month later. Müller (1997) reported that the changes in the vitamin C content of *R. villosa* followed a Gauss curve during ripening, while Uggla (2004) found a linear connection for *R. spinosissima*. The present results are in contradiction with the literature. The vitamin C content of the morphological varieties of *R. canina* did not change greatly during ripening, while the changes observed for *R. inodora* followed a saturation curve. In the fruits of *R. corymbifera* and *R. rubiginosa,* after the first vitamin C peak was reached, a further increase could be observed (Fig. 1).

The fruits of different species accumulated carbohydrates at different rates during ripening (Figs. 2 and 3), thus confirming the findings of Uggla (2004) and Uggla et al. (2005). The present investigations are in agreement with the data of Uggla (2004), who reported that the fructose content peaked a week earlier (Fig. 3) than the glucose content (Fig. 2), suggesting that the two carbohydrates are formed independently of each other in rosehips. In Sweden the carbohydrate level increased in *R. rubiginosa* until early October and in *R. dumalis* until mid-September. The sugar content always showed a linear increase as ripening progressed, until the weather cooled down in autumn, after which the levels fluctuated. The glucose and fructose contents exhibited a similar rate of change during ripening. In *R. rubiginosa* these changes followed a saturation curve, while in *R. inodora, R. corymbifera* and the mixed prickle variety of *R. canina* they followed a Gauss curve.

Conclusions

In a breeding programme underway for more than 10 years, the selection of candidate cultivars from the species *R. inodora*, *R. corymbifera*, *R. rubiginosa* and *R. canina* has now begun. The flowering, yielding and growth properties of the taxa are being evaluated to judge their suitability for use in commercial orchards. To determine the optimal harvest time the physical and chemical changes taking place during ripening are being monitored.

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SPME-GC/MS IDENTIFICATION OF AROMA COMPOUNDS IN ROSE FLOWERS

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The content and composition of active ingredients and essential oils in medicinal and aromatic plants have been studied for several decades. The volatile compounds in essential oils have been analysed routinely using gas chromatography (GC) since 1966, and with GC coupled to mass spectrometric detection (GC/MS) since 1978.

The 13 rose varieties selected for chemical analysis varied for colour, shape and fragrance. The static headspace solid phase microextraction (sHS-SPME) technique recently developed for sample preparation and sample enrichment was used to study the volatile aromatic components.

The main volatile compound of a sweet-smelling purple rose was found to be phenyl ethyl alcohol (33–52%). The phenyl ethyl alcohol content of fragrant rose flowers with blackish-purple petals increased continuously from early summer to late autumn (from 17 to 70 %). The dominant aromatic components of the yellow, orange and pink rose flowers were hexanol, hexenyl acetate and benzyl alcohol. Phenyl ethyl alcohol and orcinol dimethyl ether were the main constituents of the fragrant pink and white rose varieties. Methyl vinyl anisol and orcinol dimethyl ether were dominant in rose flowers with beige petals. In summary, it can be concluded that the SPME-GC/MS method is suitable for the characterization of rose varieties and for the chemical analysis of aromatic volatile compounds.

Key words: rose cultivars, rose petals, aromatic compounds, SPME-GC/MS method

Introduction

The genus *Rosa* includes 100–200 species and more than 18,000 cultivars and hybrids. These perennial plants are widely cultivated for their beauty and fragrance. The stems are often armed with sharp prickles. Most are native to Asia, with smaller numbers of species native to Europe, North America and Northwest Africa (Lawrence, 1991).

The flowers of *Rosa damascena* were considered to be very valuable and important in Islamic areas. The famous Persian scientist, Ibn Síná (Avicenna, 980–1037 A.D.), invented the process of distillation and was the first to distil oil from roses. The ancient Greeks and Romans used roses for healing and as a perfume component in cosmetics. Egyptians made fragrant extracts from rose petals and used them on the head, neck and face (Héthelyi and Domokos, 2000).

Roses are ancient symbols of love and beauty. They are often used as a symbol of pureness, innocence and charity (Virgin Mary, Saint Elizabeth). The Latin phrase "sub rosa" is used to denote secrecy or confidentiality. Early Christians associated the five petals of the rose with the five wounds of Jesus Christ. The red rose was eventually adopted as a symbol of the blood of Christian martyrs. In Asia and in the Middle East, however, rose oil is widely used to increase sexual desire (Jalali-Heravi et al., 2008).

Rose oil is one of the most expensive volatile oils (2.3 g = 39.0 US$), because the petals of 60,000 rose flowers are needed to produce a single ounce (28.35 g) of essential oil by steam distillation. The characteristic compounds of eastern attar are citronellol, geraniol, citral, citronellyl acetate, eugenol, ethanol, nerol, nonanol, nonanal and phenyl acetaldehyde. The health benefits of the essential oil can be attributed to its antidepressant, antiphlogistic, antiseptic, antispasmodic, antiviral, aphrodisiac, astringent, bactericidal, haemostatic, nervine, hepatic, stomachic and uterine properties (Bakkali et al., 2008).

In spite of the large number of rose species and cultivars, the flowers of only four species (*Rosa damascena* Mill., *R. gallica* L., *R. moschata* Herrm. and *R. centifolia* L.) are suitable for the production of high quality essential oils (Tucker and Maciarello, 1988). The characteristic essential oil composition of *R. damascena* grown in Isparta, Turkey was found to change during the cultivation period. The highest essential oil yield (0.040 %), with significant contents of geraniol, nerol and phenyl ethyl alcohol, was measured in May. The essential oil yield decreased in June and citronellol and linalool became the main compounds. Freshly collected petals were fermented in sacks at 25°C for various lengths of time (0, 12, 24 and 36 h). As the duration of fermentation increased, the essential oil content gradually decreased. The most significant changes during fermentation were observed in the citronellol and geraniol contents (Baydar and Baydar, 2005). The procedure used for essential oil production is very important, because it determines the volatile composition. Attar, or rose otto, is produced by steam distillation and is dominated by citronellol and geraniol. Solvent extraction yields rose absolute, the main compound of which (75%) is phenyl ethyl alcohol (Ulusoy et al., 2009).

Phytochemical studies on rose oils from Bulgaria and Damascus were performed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS). The essential oil from Bulgaria contained 95% phenyl ethyl alcohol, while the main constituents of rose oil from Damascus were citronellol, geraniol and nerol (Szabó and Héthelyi, 2008).

The static headspace solid phase microextraction (sHS-SPME) technique recently developed for sample preparation and sample enrichment was used for the investigation of the volatile aromatic components. SPME is a very simple, fast sample preparation method that can be automated and requires only small amounts of sample (0.2–0.3 g of plant material). Preliminary studies on aromatic leaf, seed and root samples showed that the method is suitable for the identification and chemical characterization of volatile constituents and can replace the time-consuming hydrodistillation (Héthelyi et al., 2009).

The aim of the present work was to characterize the chemical composition of volatile components in the petals of 13 selected rose varieties with different colour, shape and fragrance during the cultivation period (May to November). The volatile aromatic compounds were studied using the sHS-SPME-GC/MS technique.

Materials and methods

Plant material

The 42–45 rose cultivars supplied by Margit Ambrus, Jusztinia Kukuly (Szentendre) and Gergely Márk (Törökbálint) have been cultivated in a private garden in Budapest since 1970. The rose varieties were selected for the colour, shape and fragrance of the flowers, the height of the stems, the shape and sharpness of the prickles, the frequency of flowering, and disease resistance. The 13 most valuable cultivars were used for the chemical analyses. The samples were collected on sunny days at noon.

Solid phase microextraction (SPME)

Fresh or air-dried rose petals (3–5) were put into vials (20 mL headspace) sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 μM StableFlex polydimethyl siloxane/divinyl benzene (PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 60°C, extraction was performed by exposing the fibre to the headspace of a 20 mL vial containing the plant material for 10 min at 60°C. The fibre was then immediately transferred to the injector port of the GC/MS, and desorbed for 1 min at 250°C. The SPME fibre was cleaned and conditioned in a Fibre Bake-out Station in pure nitrogen atmosphere at 250°C for 15 min after desorption.

Apparatus and GC-MS conditions

The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent HP-5MS capillary column (30 m × 250 μm × 0.25 μm). The GC oven temperature was programmed to increase from 60°C (3 min isothermal) to 200°C at 8°C/min (2 min isothermal), from 200–230°C at 10°C/min (5 min isothermal) and finally from 230–250°C at 10 °C/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. The injector temperature was 250°C and the split ratio was 1:50.

The mass selective detector was equipped with a quadrupole mass analyser and was operated in electron ionization mode at 70 eV in full scan mode (41–500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 05 library was also consulted.

The percentage data of the total ion current chromatograms were calculated with the area normalization method without applying a response factor correction. The percentile values of the components represent their distribution in the volatile part of rose petals.

Results and discussion

The 13 rose cultivars studied had diverse colour and fragrance. The colour of the petals ranged from blackish-purple and dark red to light purple, pink, orange, yellow, beige, white and pink with purple spots.

The main volatile compound of the sweet-smelling dark purple rose was found to be phenyl ethyl alcohol (33–52%). It also contained citronellol, phenyl ethyl acetate, heptadecane, nonadecane and nonadecene. The methyl geranate content changed significantly with the cultivation time (2–22.9%), the largest quantity occurring in October.

The phenyl ethyl alcohol content of the fragrant rose flowers with blackish-purple petals increased continuously from early summer to late autumn (from 17 to 70%). The minor constituents were found to be benzaldehyde, hyacinthine, citronellol, geraniol, phenyl ethyl acetate, nonadecene and nonadecane. The methyl geranate content changed significantly during the cultivation period (2–20%), the largest quantity occurring in September. The nonadecane and nonadecene contents were also highest in autumn (19 and 10.4%, respectively).

The main volatile compounds of the yellow, orange and pink rose flowers were hexanol (5.8–37.2%), hexenyl acetate (18–47.7%) and benzyl alcohol (16.1–45.1%).

The orange rose flowered throughout the cultivation period. The aromatic constituents were monitored from June to October. Hexanol (5.8–37.2%), benzaldehyde (3.8–20.5%), hexenyl acetate (18.1–68.9%) and benzyl alcohol (15.8–45.1%) were the characteristic components.

Phenyl ethyl alcohol and orcinol dimethyl ether were the main constituents of the fragrant pink (30 and 38.6–67.4%, respectively) and white rose varieties (47.9–77.7% and 17.7–44.4%, respectively). Phenyl ethyl alcohol is characteristic of the rosy scent of Hybrid Tea roses, while orcinol dimethyl ether (3,5-dimethoxy toluene) provides the typical "tea" scent (Cherri-Martin et al., 2007). The most beautiful flowers were obtained in mid-June and late September.

Methyl vinyl anisol (16.1–79.2%) and orcinol dimethyl ether (84.1–20.3%) were dominant in rose flowers with beige petals. The methyl vinyl anisol content increased with the cultivation time, while the orcinol dimethyl ether quantity decreased significantly.

The main aromatic components of a less intensively fragrant yellow rose were citronellol (9.4–15.4%), geraniol (14.8–15.7%), orcinol dimethyl ether (41.9–48.3%), nonadecene (10.1–12.1%) and nonadecane (2.9–3.9%). Earwigs (*Forficula auricularia*) were often found in these flower samples, so harvested plants were left in the garden for one or two hours.

The phenyl ethyl alcohol content of fragrant, light purple rose flowers increased continuously from July to September (25.1–53.4%). The orcinol dimethyl ether amount decreased significantly (52.1–9.4%) until October, when it suddenly increased to 77.9%, because of the hexanol content was higher (17.4%) in October.

The main volatile aromatic compounds of fragrant yellow rose petals with pink edges were hexanol (18.4–24.6%), hexenyl acetate (36.7–47.7%) and benzyl alcohol (16.1–22.7%).

The fragrant yellow rose petals contained hexanol (9.9–11.1%), hyacinthine (1.6–5.6%), citronellol (4.9–11.6%), thymol methyl ether (1–2.2%), geraniol (1–23.7%) and orcinol dimethyl ether (27.5–28.1%).

In summary, it can be concluded that the sHS-SPME-GC/MS method was suitable for the isolation and chemical characterization of aromatic volatile compounds in rose petals. The rose samples contained numerous volatile compounds and about 30 specific constituents were identified. The white and light purple roses were found to be Hybrid Tea roses. During the cultivation period significant changes were observed in the percentile distribution of the volatile compounds.

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DIFFERENT RESPONSES OF SENSITIVE AND RESISTANT APRICOT GENOTYPES TO ARTIFICIAL *Monilia laxa* (ADERH. & RUHL.) INFECTION

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Blossom blight caused by *Monilia laxa* (Ehr.) is the most important fungal disease in Hungarian apricot orchards. The cultivars traditionally grown in the country are susceptible to *Monilia laxa* (Ehr.) to various extents. In this study the shoots of one tree each of the varieties Zard and Korai Zamatos and 48 hybrids from their cross were artificially infected *in vivo* with *Monilia laxa* (Ehr.). The results indicated that when artificial infections are made to evaluate pathogen resistance, this should be carried out on one-year-old shoots, since this is the natural infection point of *Monilia*. It also appears that, due to the great variability in the size of destroyed tissues, the microscopic analysis of the infections could provide a more reliable evaluation of progeny resistance than comparing the sizes of destroyed shoot areas.

Key words: *Monilia* resistance, apricot, artificial infection, blossom blight

Introduction

Blossom blight caused by *Monilia laxa* (Ehr.) in apricot is one of the most destructive fungal pathogens in Hungary. This was already recognised in the first apricot breeding programme in Hungary, launched by Gyula Magyar in the 1930s, the main aim of which was to increase resistance to frost and blossom blight (Pedryc, 2003). Blossom blight results in losses in productivity and shortens tree longevity. Chemical treatment against blossom blight during the flowering period is expensive, causes chemical pollution and is often not sufficient to defeat the infection. For example, in 2004 three fungicide applications were necessary in flatland areas and two in hilly regions to decrease the damage caused by this disease, and full prevention was not still achieved (Drén et al., 2007).

Most of the information available on varietal susceptibility is based on orchard observations according to which differences between apricot genotypes exist, but no absolutely resistant genotype was detected (Gutermuth and Pedryc, 2009). The unreliability of field evaluation was also emphasised by Crossa-Raynaud (1969). To avoid false conclusions, observations must be repeated over 3–4 years if a valid disease rating is to be obtained. Long-term monitoring (five years) decreases the influence of annual changes in weather conditions and allows cultivars to be evaluated on the basis of genetically determined differences. Although field observations are the most important to decide if a genotype is resistant or not, a reliable, rapid artificial method is needed for the evaluation of new hybrids. Such a method was described by Crossa-Raynaud (1969), and this was subsequently applied by Gulcan et al. (1997) in Turkey, Nicotra et al. (2006) in Italy and Trandafirescu and Teodorescu (2006) in Romania. This method involves the inoculation of 2–4-year-old branches removed from the tree under controlled conditions. In contrast, Harada et al. (2004) inoculated fruit-bearing branches of *Prunus mume* (Sieb. & Zucc.) to evaluate the virulence of different *Monilia* species, and all three species studied caused lesions on the shoots. The data included longitudinal measurements on the rotten phloem tissue and the classification of the length. The relative susceptibility of the cultivars was the same in winter and spring tests and both were in agreement with field observations. None of these studies detected fully resistant apricot genotypes; in all cases tissue canker was observed. A study on the resistance of progenies derived from resistant parents by open pollination showed that resistance is a quantitative genetic trait (Nicotra et al., 2006). Nevertheless the same authors emphasised that the trait was inherited in a fundamentally dominant way. Crossa-Raynaud (1969) evaluated 45 hybrids derived from the resistant Hamidi × susceptible Canino cultivars. In terms of susceptibility to brown rot the progenies were evenly distributed between the two parents. The preponderance of individuals almost as resistant as the Hamidi parent would seem to signify the genetic dominance of the gene or genes for resistance.

Developing a disease-resistant cultivar would be the most effective and safe solution for plant protection, both for environmental and economic reasons (Fred et al., 2007).

Materials and methods

Plant material and infection method

The susceptible Korai Zamatos and field-resistant Zard varieties were crossed in 2003, and 48 progenies were grown in the experimental orchard of the Department of Genetics and Plant Breeding, Corvinus University of Budapest, at Soroksár. The parents and each of the seven-year-old seedlings were inoculated through fifteen and eight 2–5 mm diameter slits, respectively, cut with a lancet on the shoots opposite the buds. Potato dextrose agar (PDA) discs, 3 mm in diameter and infiltrated with *Monilia laxa* (Ehr.), were inserted in the wounds. The surface and the lancet

were disinfected with 70% ethanol. The wounds were then covered with parafilm. As a negative control, one wound on each tree was filled with sterile PDA (without mycelium). The wounds were continuously monitored and the evaluation of infection was carried out in January, ten weeks after inoculation. The infected shoots were excised and the length of destroyed tissue was measured with a Vernier caliper after the bark was peeled. The sections were then studied and photographed using a stereomicroscope. The twig diameter was measured at the infection point. The correlation between the length of destroyed tissue and the diameter of the infected branches was calculated. Each infection length was divided by the diameter of the branch to avoid errors due to different branch diameters.

Fungal material

The fungal material was collected from strongly sporulating, mummified apricot fruit.

The conidia were directly transferred from infected tissues onto fresh potato dextrose agar (200 g potato, 10 g dextrose, 20 g agar powder, 1000 ml distilled water; hereafter, fresh PDA) with a sterile needle, then cultivated in Petri dishes on shallow (2–3 mm) fresh PDA medium at 20°C in the dark for ten days.

Statistical analysis

All statistical tests were performed using Statistica 8.0 (Statsoft, 2007) software. Assumptions (normality and homogeneity of variances) were checked previous to every test, and where the assumptions did not meet, non-parametric statistical tests were utilized.

Results

When the mean length of infected tissue was compared for Zard and Korai Zamatos, higher values were surprisingly detected on the field-resistant variety Zard (Fig. 1). However, the *t-test* showed no significant difference between the average infected shoot lengths. On the microscope photographs, two clearly distinguishable tissue responses were observed (Fig. 2). In the case of the sensitive parent, Korai Zamatos, the border between the destroyed and unharmed tissue was indistinct. On the resistant variety, Zard, the unharmed tissue was sharply separated, with a thickened edge. The same responses were detected on the progenies. Based on this observation, 17 of the progenies were ranked in the resistant group, and 31 in the susceptible group. In both cases the fungus could be isolated from the infected parts of all the plants. When the shoot diameter was less than 2 mm, the pathogen completely surrounded the phloem. These samples were excluded from the survey. On the negative control slides no differences were observed between the two phenotypes. The wounds exhibited a thickened edge, as observed for resistant genotypes.

The average length of infected tissue was calculated for each progeny and was found to be significantly different (8.6 and 5.1 mm, respectively) for resistant and susceptible progenies (Mann-Whitney test $Z=1.9858$, $p=0.047$ and Welch-test $d=2.838$, $p=0.007$) (Fig. 1).

The infected branch length was significantly correlated with the diameter of the infected branch (Spearman correlation $r=0.0458$, $p=0.12$).

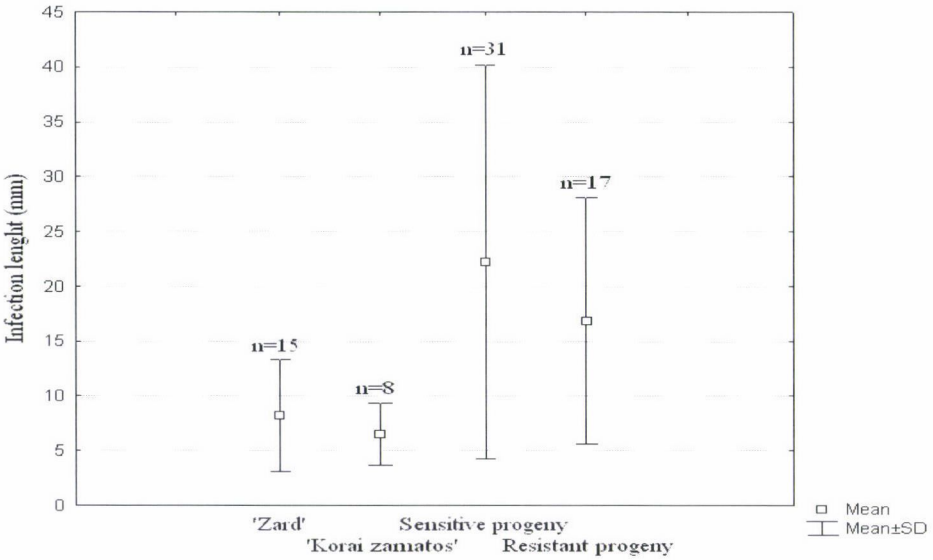

Fig. 1. Comparison of the infected shoot lengths of the two parent varieties, Korai Zamatos and Zard, and their hybrid progenies. The hybrids were divided into susceptible and resistant groups based on their microscopic tissue morphology. In the case of the parents, n refers to the number of infections per plant and in the case of the progenies to the number of infected plants

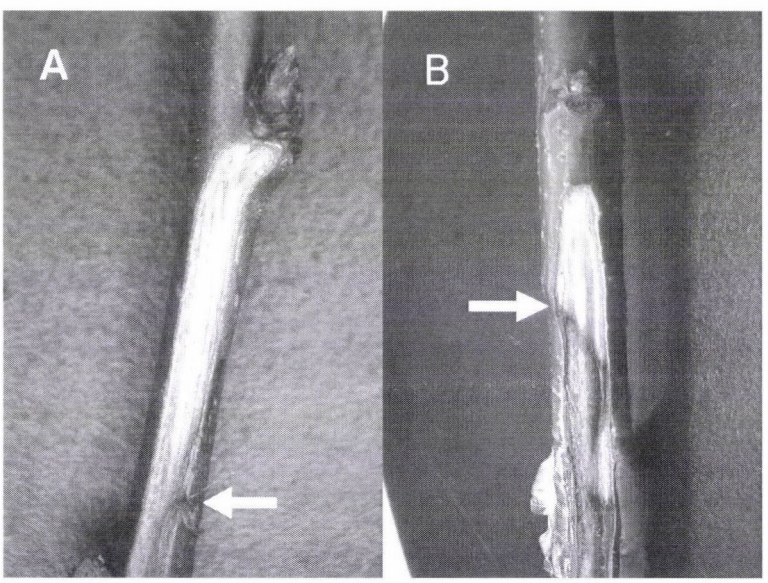

Fig. 2. A: Korai Zamatos shoot infected with *Monilia laxa* (Aderh. & Ruhl.). Arrow shows the indeterminate boundary of the dead tissue. The dark lesion of sieve elements shows the continuous destruction caused by the pathogen. B: Zard shoot response to infection with *Monilia laxa* (Aderh. & Ruhl). Arrow shows the distinct boundary between intact and dead tissue. The inviolate part is thickened

Conclusions

The Korai Zamatos apricot variety was previously described as susceptible and the Zard variety as resistant to blossom blight (Gutermuth and Pedryc, 2009). In this study shoots of one tree each of Zard and Korai Zamatos and of 48 hybrids from their cross were artificially inoculated *in vivo* with *Monilia laxa* (Ehr.). Previously the evaluation of *Monilia laxa* (Ehr.) susceptibility had only been performed *in vitro* on excised shoots.

The aim was to survey the interaction between the necrotrophic pathogen and the apricot plant *in vivo*, in order to examine the effects of *Monilia* in its natural environment. While Crossa-Raynaud (1969) and other researchers using the same method tested excised 3–4-year-old branches, the present investigation was carried out on fruit-bearing shoots where flower buds develop. The monitoring of natural infection has previously shown that only one-year-old shoots are subject to fungal injuries. In the case of infection on the shoot, the fungal destruction stops at the boundary between the fruit-setting shoot and the older branch, leaving a wound on the surface of the twig, as reported by Holb (2003). Thus, it was chosen to test susceptibility on fruit-setting shoots rather than on older branches, as done in previous studies. After artificial inoculation, Trandafirescu and Teodorescu (2006) reported high variance in the size of the destroyed tissue, but this was not found by Crossa-Raynaud (1969), Gulcan et al., (1998) or Nicotra et al. (2006). The Crossa-Raynaud technique evaluates susceptibility or resistance based on the length of the infected area. In the present study this comparison gave unexpected results for Zard and Korai Zamatos, since the average length of destroyed tissue was greater in Zard, though this difference was not statistically significant. Thus, the evaluation of the size of the destroyed area after artificial infection did not reveal the difference between the two varieties previously reported by Gutermuth and Pedryc (2009).

In order to find a trait that consistently differed for infections on susceptible and resistant varieties, microscope sections were made. When these were examined under a stereomicroscope two clearly distinguishable phenotypes of plant response to the pathogen were observed. One morphological type could be detected in each infection on the resistant variety Zard, while the other type occurred on every infected shoot of Korai Zamatos. On the basis of the microscope investigation, the 48 Zard × Korai Zamatos hybrids were found to consist of 31 susceptible and 17 resistant genotypes. The proportion of susceptible and resistant phenotypes among the hybrids was in contrast to previous studies by Crossa-Raynaud (1969), Gulcan et al. (1999) and Nicotra et al. (2006), where the majority of the offspring were almost as resistant as the resistant parent. Due to the low number of offspring, no attempt was made to calculate the segregation ratio, and it is clear that the inheritance of *Monilia* resistance cannot be clarified on the basis of these results. It would appear, however, that resistance to *Monilia* is not a dominant trait. After calculating the average length of destroyed tissue for each hybrid, those classified as susceptible had significantly higher infected shoot lengths than those classified as resistant. This confirms the expected outcome from the Crossa-Raynaud technique. In

each of the apricot plants surveyed the length of dead tissue showed high within-plant variability (Fig. 1), and a significant negative correlation was found between the destroyed tissue length and the diameter of the twigs. This suggests that the infection is stronger on thinner shoots.

Fruit breeding is a difficult task, mainly because of the long juvenile phase of the trees and the very high level of heterozygosity of most fruit species. The development of a new technique applicable for the early and reliable selection of seedlings carrying valuable traits has become a priority in fruit breeding. It could be concluded that when artificial inoculation is used to evaluate pathogen resistance, this should be carried out on one-year-old shoots, since this is the natural infection point of *Monilia*. It is also suggested that, due to the high variability in the size of destroyed tissues within each plant, microscopic analysis could be a more reliable way of evaluating progeny resistance than comparing the sizes of destroyed shoot areas.

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GOALS, PRESENT POSITION AND PROSPECTS OF FORAGE SORGHUM BREEDING IN HUNGARY

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Forage sorghum species, comprising grain and silage sorghum (*Sorghum bicolor)* and sudangrass *(Sorghum sudanense*), are considered the most drought-tolerant field crops in Hungary (Siklósiné Rajki, 1997). Several features, such as its tropical origin, deep voluminous roots, and thick waxy leaves and stem, enable the plants to survive periods with insufficient rainfall (Wall and Ross, 1970). Owing to its large roots, the crop shows wide adaptability to soils with moderate or low fertility. Though sorghum is used mainly for forage in Hungary, it is a very important source of bioenergy in other countries. Hungary lies in the northern part of the European sorghum belt, so the emphasis in the breeding programme is to develop early or mid-early forage sorghum hybrids adapted to Hungarian climatic conditions and soil properties, to introduce them into commercial production and to make them available for farmers.

Key words: sorghum, breeding, drought tolerance, forage, bioenergy

Introduction

Sorghum breeding started in Hungary in the 1950s (Barabás, 1958), and it was first produced in the 1970s, once the agrotechnical background (chemical weed control, suitable machines for sowing, harvesting and drying) had been developed in Hungary. Since then forage sorghum has been produced as fodder, but there is now serious interest in using it for bioenergy.

Materials and methods

The testing of novel sorghum germplasm is carried out in various locations. In 2009 homogeneous inbred R-lines, CMS A and maintainer B lines, segregating lines and observation trials were planted on alkaline soil in Kiskundorozsma in early May. Comparative trials were set up on two different soil types (sandy and alkaline soil) on 20 m^2 plots in a randomised block design with four replicates to test the adaptability of the hybrids.

The soil surface was compacted with a field roller after planting and the herbicides RAMROD FLO and PLEDGE 50 WP were sprayed pre-emergence to control mono- and dicotyledonous weeds. Later the experimental areas were kept free of weeds mechanically. The following traits were recorded: time of emergence; early vigour; time of panicle emergence and 50% flowering; panicle length and diameter; neck length; extent of MDMV infection and uniformity.

The panicles were isolated individually at emergence to ensure the homogeneity of the sorghum lines, using parchment bags for fertility-restoring inbred lines and segregating lines and cellophane bags for CMS female lines. In the advanced stage of flowering the CMS (A) lines were crossed under the cellophane bags, either with new fertility-restoring (R) lines, developed during 5–6 years of inbreeding, or with maintainer (B) lines to maintain sterility. The isolated panicles were individually harvested and screened by visual evaluation.

Besides being grown for forage, silage sorghum is also an important crop for biogas production. Due to the increased importance of energy crops, the biogas yield of the experimental silage sorghum hybrids was analysed in cooperation with other institutions. Small-scale silage samples were manufactured for novel hybrids exhibiting high yield and sugar content and excellent stem strength at the Faculty of Agriculture of the University of Szeged in Hódmezővásárhely and the biogas yield was assessed in the laboratory of the Hungarian Institute of Agricultural Engineering of the Ministry of Agriculture and Rural Development. A total of 12 samples were analysed.

Few herbicides are available for chemical weed control in sorghum. One reason may be that forage sorghum is a minor crop in Hungary, grown on only 20–25 thousand ha annually. Trials have been conducted since 2005 to test the herbicides authorised for other crops in sorghum. These tests are carried out on 10 m^2 plots in a randomised block design with four replicates and under large-scale conditions (0.1 ha), using pre-emergence and post-emergence herbicides for the control of mono- and dicotyledonous weeds.

Results and discussion

In 2009, 400 crosses (250 in grain sorghum and 150 in silage sorghum) were made to develop experimental hybrids that will be planted in observation and comparative trials in 2010.

The grain and silage sorghum hybrids bred in earlier years were planted in comparative trials and evaluated after harvesting. The experimental grain sorghum hybrids 15/08, 26/08, 25/08, 16/02 and 16/08 gave the best results on alkaline soil (Table 1), but these are later than the standards (GK Emese and Alföldi 1). Only 15/08 was better than the standard, GK Emese, on sandy soil (Table 2). This experimental hybrid performed well in both experiments, so it appears to be promising for the future.

Among the experimental silage sorghum hybrids, 20/08 and 76/06 gave the highest green mass on both alkaline and sandy soils (Tables 3 and 4). These hybrids are later than the standard, Róna 1, but they could be promising and their testing will be continued in the following years.

The biogas production of maize and sorghum varieties was examined at the Hungarian Institute of Agricultural Engineering of the Ministry of Agriculture and Rural Development in Gödöllő (Table 5). The results show differences between the varieties in biogas yield, indicating the necessity to breed species and varieties especially for high biogas production (Pál and Rajki, 2008; Pál et al., 2007).

Table 1
Grain sorghum experiment on alkaline soil (Kiskundorozsma, 2009)

Variety	Flowering date	Plant height (cm)	1000-seed weight (g)	Grain yield (t/ha)
15/08	24 July	125.0	33.0	10.45
26/08	24 July	115.5	31.6	9.95
25/08	22 July	100.5	30.0	9.07
16/02	23 July	112.5	26.4	8.57
16/08	28 July	117.5	28.2	8.16
Alföldi 1 st.	20 July	140.0	26.2	7.59
GK Emese st	17 July	150.0	25.4	7.20
28/08	25 July	100.5	26.0	7.10
24/08	20 July	102.5	25.3	6.92
33/06	20 July	105.0	25.8	6.40
14/08	20 July	100.0	30.2	6.23
Average	21 July	115.3	28.0	7.97
$LSD_{5\%}$				0.75

Table 2
Grain sorghum experiment on sandy soil (Kiskundorozsma, 2009)

Variety	Flowering date	Plant height (cm)	Grain yield (t/ha)
Alföldi 1 st.	24 July	130	6.39
15/08	25 July	95	5.92
GK Emese st.	23 July	135	5.13
16/08	31 July	110	4.68
14/08	26 July	120	4.42
28/08	30 July	95	4.30
25/08	25 July	90	3.60
16/02	18 July	105	3.56
26/08	26 July	105	3.39
33/06	24 July	95	2.51
24/08	25 July	90	2.42
Average	25 July	106	4.21
$LSD_{5\%}$			0.91

Table 3
Silage sorghum experiment on alkaline soil (Kiskundorozsma, 2009)

Variety	FD	PH	GMY	SCW	SCR
Exp 20/08	2 August	210	51.4	15.0	10.5
Exp. 76/06	5 August	220	50.5	16.0	10.0
Róna 1 st.	26 July	200	49.3	17.0	13.0
Exp. 77/06	1 August	195	47.9	14.5	11.5
Exp. 13/06	22 July	190	36.4	16.0	15.0
Average	29 July	203	47.1	15.7	12.0
$LSD_{5\%}$			6.0		

FD: Flowering date, PH: Plant height (cm); GMY: Green mass yield (t/ha); SCW: Sugar content in waxy stage of grain (refraction %); SCR: Sugar content in ripe grain (refraction %)

Table 4
Silage sorghum experiment on sandy soil (Kiskundorozsma, 2009)

Variety	FD	PH	GMY	SCW	SCR
Exp. 76/06	10 August	210	41.4	17.0	10.0
Exp 20/08	10 August	205	39.0	15.0	10.5
Róna 1 st.	31 July	190	34.6	17.0	15.5
Exp. 77/06	4 August	180	27.5	18.0	14.5
Exp. 13/06	28 July	180	21.7	19.5	15.5
Average	4 August	193	32.8	17.3	13.2
$LSD_{5\%}$			8.3		

FD: Flowering date, PH: Plant height (cm); GMY: Green mass yield (t/ha); SCW: Sugar content in waxy stage of grain (refraction %); SCR: Sugar content in ripe grain (refraction %)

Table 5
Analysis of biogas yield
(Hungarian Institute of Agricultural Engineering of the Ministry of Agriculture and Rural Development)

Variety	DM	OM	NC	CC	SGP	MP
Silage of Róna 1 sorghum hybrid (Kiskundorozsma)	27.0	95.0	4.18	225.45	415.8	257.8
Mixed silage of Róna 1 sorghum + Kenéz maize hybrid (Kiskundorozsma)	31.0	96.0	5.16	225.15	396.7	245.9
Mixed silage of Róna 1 sorghum + Kenéz maize hybrid (Újszeged)	36.0	89.0	6.29	229.57	391.4	242.7
Silage of Róna 1 sorghum hybrid (Újszeged)	32.0	85.0	5.94	227.48	361.2	224.0
Silage of Kenéz maize hybrid (Kiskundorozsma)	39.0	96.0	5.81	222.28	308.9	191.5
Silage of EXP06 experimental sorghum hybrid (Kiskundorozsma)	30.0	96.0	4.62	222.64	296.1	183.6

DM: Dry matter content (%); OM: Organic matter content (%); NC: Nitrogen content (%); CC: Carbon content (%); SGP: Specific gas production (NL/kg organic dry matter); MP: Methane production (NL/kg organic dry matter)

In the weed control trial, chemicals suitable for controlling weeds in sorghum were identified, which can be substituted for herbicides containing atrazine as active ingredient, the use of which has been banned in the EU. One of the herbicides, Pledge, has recently been registered and is authorised for farm use in Hungary (Pál, 2009).

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IN VITRO AND *IN VIVO* SCREENING FOR DROUGHT TOLERANCE IN WINTER × SPRING WHEAT DOUBLED HAPLOIDS DERIVED THROUGH CHROMOSOME ELIMINATION

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The relative efficiency of *in vitro* and *in vivo* screening techniques for drought tolerance, comprising various parameters, namely germination (%), shoot length, root length, coleoptile length, root number, root/shoot ratio and seedling vigour index (SVI) under *in vitro* conditions and morpho-physiological and yield-contributing traits under *in vivo* conditions, was studied using 78 winter × spring wheat-derived doubled haploid lines of bread wheat along with 13 parental genotypes and two check varieties, HPW 155 and PBW 343. Analysis of variance for different *in vitro* parameters in control (0 MPa) and stress (–0.7 MPa) environments and various *in vivo* parameters under irrigated and rainfed environments indicated sufficient genetic variability and the differential response of the genotypes to the different stress levels for all the *in vitro* and *in vivo* parameters. Correlation studies revealed the significance of percentage germination, root number, coleoptile length and seedling vigour index under *in vitro* conditions and relative water content and excised leaf water loss under *in vivo* conditions as important selection criteria for drought tolerance, as these parameters were related with each other as well as with the drought susceptibility index (S). The significant positive rank correlation between the *in vitro* (–0.7 MPa) and *in vivo* (rainfed) stress conditions indicated that the performance of a genotype under field conditions is very similar to its performance under laboratory conditions. Hence, the selection precision for a crucial and complex trait like drought tolerance in wheat can be enhanced by exercising *in vitro* selection coupled with evaluation in the field. The drought susceptibility index 'S' should not be taken as the sole criterion to categorize genotypes as drought-tolerant or susceptible ones.

Key words: drought screening, doubled haploids, winter × spring wheat hybridization, wheat × maize system, *in vitro* screening, *in vivo* screening

Introduction

Improving the drought tolerance of crops is a major objective of most plant breeding programmes as drought stress is one of the major constraints limiting wheat productivity. The frequent occurrence of dry spells, generally at

the early growth and post-anthesis stages, has been recognized as affecting the stability and sustainability of wheat production in most regions of the world. This necessitates the development of wheat varieties with higher yield stability and potential and the identification of parameters that confer drought tolerance on the plants, so as to increase the yields in dry areas. One of the most promising breeding approaches to achieve stability under drought is to exploit the available diversity of the winter wheat gene-pool. This gene-pool is known to carry desirable genes for tolerance/resistance to drought and other abiotic and biotic stresses, besides having high yield potential and can be utilized for the genetic improvement of the predominantly grown spring wheat genotypes. Thus, such a breeding approach has great potential to generate a wide range of high-yielding recombinants resistant to drought and other stresses.

To develop wheat varieties with high yield in drought-prone areas, it would be desirable first to isolate homozygous lines from winter × spring wheat F_1 hybrids and then to subject them to various *in vitro* and *in vivo* screening processes to improve selection efficiency for drought tolerance. The chromosome elimination-mediated approach, using the wheat × maize system, has proved to be a potent tool to obtain instant homozygous lines (doubled haploid, DH) from winter × spring wheat F_1 hybrids in the shortest possible time (Chaudhary et al., 2002; Sharma et al., 2005).

The influence of various morphological, physiological and agronomic characters, as well as traits such as leaf morphology (area, shape, waxiness, senescence and pubescence), stomata control (number, size, aperture), root characteristics, relative water content, excised leaf water loss, index of drought resistance, coleoptile length, seedling vigour index, grain yield and yield components, abscisic acid level, proline content, dehydrin accumulation, cell membrane stability, etc. on drought tolerance have been studied by various workers (Cedola et al., 1994; Aydin et al., 2000; Alderfosi, 2001; Deora et al., 2001; Farooq and Azam, 2001; Kinyua et al., 2003; Lopez et al., 2003; Larbi and Mekliche, 2004) for evaluating drought tolerance. Field evaluation for drought tolerance may be difficult, as the crop may be exposed to various levels of water deficit at different growth stages (Hsiao et al., 1976) and is often complicated by G × E interactions. Such interactions may be further complicated due to unpredictable weather conditions such as excessive rain during the crop season, flooding, moisture content in the soil and also on account of various diseases. In such situations, *in vitro* screening can help to overcome the problems associated with the inconsistency and unpredictable onset of stresses and the confounding effects of other stresses. Therefore, the present investigation was undertaken to screen winter × spring wheat-derived doubled haploids for drought tolerance under *in vitro* and *in vivo* conditions, to correlate the field performance of the selected doubled haploid lines with their *in vitro* performance and to identify the major characteristics that would improve selection efficiency for drought tolerance.

Materials and methods

The material for the study comprised 78 DH lines derived from 21 elite and diverse winter × spring wheat F_1 hybrids, namely Saptdhara × HPW 89, Saptdhara × HPW 147, Saptdhara × HPW 184, Saptdhara × HW 3024, Saptdhara × PW 552, Saptdhara × UP 2418, Sentry × HPW 89, Sentry × PW 552, Sentry × UP 2418, VWFW 452 × HPW 42, VWFW 452 × HPW 89, VWFW 452 × HW 3024, VWFW 499 × HPW 147, W 10 × HPW 42, W 10 × HPW 89, W 10 × HW 3024, W 10 × PW 552, W 10 × UP 2418, WW 24 × HW 3024, WW 24 × PW 552 and WW 24 × UP 2418 following the standard (Laurie and Bennett, 1988) and refined protocols (Chaudhary et al., 2002; Sharma et al., 2004) of the chromosome elimination-mediated approach following the wheat × maize system, along with the 13 parental genotypes and two check varieties, HPW 155 (a recently released wheat variety for rainfed cultivation in high hill areas) and PBW 343 (a popular variety for low altitude irrigated areas).

Experiment 1
In vitro *screening method*

All the DH lines, along with the parents and checks, were evaluated for various *in vitro* parameters such as germination percentage, shoot length, root length, coleoptile length, root/shoot ratio, root number and seedling vigour index on filter paper in 25 cm diameter Petri dishes at a constant temperature of 25 ± 1°C in a growth chamber. To create water stress environments, polyethylene glycol-6000 (PEG-6000) was used, following the methods of Michael and Kaufmann (1973).

The seeds of the winter wheat genotypes, Saptdhara, Sentry, VWFW 452, VWFW 499, W 10 and WW 24 and the spring wheat genotypes, HPW 42, HPW 89, HPW 147, HPW 184, HW 3024, PW 552 and UP 2418, along with the two check varieties, HPW 155 and PBW 343, were used to determine LD_{50} (dose of PEG-6000 which creates an osmotic potential at which the growth of the seedlings is restricted to 50% as compared to the distilled water control). Different concentrations of PEG-6000 dissolved in water were used to simulate drought stress at different levels of osmotic potential (–0.2, –0.3, –0.4, –0.5, –0.6, –0.7, –0.8, –0.9 and –1.0 MPa) along with control (0 MPa) conditions. A seed was considered germinated if it had at least 2 mm emergence of both radicle and plumule at the time of data recording. At –0.7 MPa osmotic potential, seedling growth was restricted by 50% as compared to the control, whereas at –0.8, –0.9 and –1.0 MPa, seed germination and seedling germination were drastically affected. However, significant differences were not observed with respect to germination and growth when the seedlings were grown at –0.2, –0.3 and –0.4 MPa osmotic potential, giving values similar to that of the control.

The experimental material was therefore evaluated under two levels of moisture stress, i.e. control (0 MPa) and stress (–0.7 MPa) conditions, in three sets. The observations on all the aforementioned *in vitro* parameters in control and stress environments for each set were recorded on the 10th day. For germination percentage, 50 surface-sterilized seeds per genotype, and for the rest of the parameters, 10 surface-sterilized seeds per genotype were placed in control (0 MPa) and stress (–0.7 MPa) environments in sterilized Petri dishes for each of the three sets. Observations pertaining to these parameters, except germination, were recorded on five randomly selected seedlings. Seedling vigour index (%) was calculated by multiplying the sum of shoot and root lengths by the germination percentage (Abdul-Baki and Anderson, 1973).

Experiment 2
In vivo *screening methods*

The *in vivo* (field) evaluation of all the 78 DH lines and the parental genotypes along with the checks was accomplished by raising these genotypes in irrigated and rainfed environments in a randomized block design with three replications. The irrigated environment was given four irrigations at the crown root initiation, late jointing, flowering and grain-filling stages, whereas no irrigation was applied to the rainfed environment. The data were recorded on five randomly selected competitive plants per genotype from each replication on the various morpho-physiological traits: plant height, flag-leaf area, relative water content and excised leaf water loss, the yield-contributing traits: effective tillers per plant, spike

length, spikelets per spike, grains per spike, grain yield per plant and 1000-grain weight, the harvest index and a drought-related trait, the drought susceptibility index (S). Data pertaining to relative water content and excised leaf water loss were recorded at anthesis, and the rest of the morpho-physiological and yield-contributing traits at maturity. A record of phenological traits such as days to flowering and days to maturity was made on a plot basis. Basic statistical parameters such as mean, range, coefficient of variation, broad sense heritability (h^2_{bs}) and genetic advance were determined. The following limits were used for categorizing the magnitude of different parameters:

	High	Moderate	Low
Phenotypic coefficient of variation (PCV)	> 20 %	10–20 %	<10%
Genotypic coefficient of variation (GCV)	> 20 %	10–20 %	<10 %
Heritability (h^2_{bs})	> 80 %	50–80 %	<50 %
Genetic advance	> 50 %	30–50 %	<30%

The drought susceptibility index (S) used to characterize the relative drought tolerance of the genotypes, based on the moderation of yield losses in a stress environment, was calculated from the grain yield data recorded under rainfed (moisture stress) and irrigated (non-stress) environments using the formula given by Fischer and Maurer (1978):

$$S = [1 - Yd / Yp] / D$$

$$D = 1 - [\text{Mean Yd of all the genotypes} / \text{Mean Yp of all the genotypes}]$$

where Yd is the mean grain yield of the variety/strain in a rainfed environment; Yp is the mean grain yield of the variety/strain in an irrigated environment and D = drought intensity.

The correlation coefficients were estimated between various *in vitro* and *in vivo* parameters in the control/irrigated and stress/rainfed environments. The correlation of the drought susceptibility index (S) with various *in vitro* and *in vivo* parameters was also studied.

On the basis of the performance of the genotypes with respect to different traits under *in vitro* and *in vivo* conditions, the genotypes were ranked for each trait pertaining to drought tolerance. Rank 1 was given to the genotype having the highest value and so on up to 93, where rank 93 was assigned to the genotype with the lowest value. A common rank was given to genotypes with the same value for a particular trait (Elhance, 1974). The overall rank of the genotype under *in vitro* and *in vivo* conditions was obtained by adding the ranks of each genotype with respect to the different *in vitro* and *in vivo* parameters under study. The coefficient of rank correlation (rs) was calculated, on the basis of the overall ranking of the genotypes, between the rainfed (*in vivo*) and *in vitro* stress (–0.7 MPa) environments so as to study the correlation of field performance with the *in vitro* results, calculated using the following formula:

$$rs = 1 - \{6[(\sum d^2) + CF] / n(n^2 - 1)\}$$

where correction factor (CF) = $1/12\ [(m^3 - m)]$; m = genotypes whose ranks are common; $\sum d^2$ = total of the squares of the differences of corresponding ranks; n = number of genotypes.

If in a series there are m genotypes whose ranks are common, a correction factor (CF) is added to the value of ($\sum d^2$) to correct the coefficient of rank correlation (rs). If there is more than one group of genotypes with common ranks, CF is added as many times as the number of such groups.

The significance of the rank correlation (rs) was tested using the following formula:

$$t = \sqrt{\frac{n-2}{1-rs^2}} \times rs$$

where: n = number of genotypes; rs = rank correlation

Significance was then tested using the t-table at (n–2) degrees of freedom.

Results and discussion

Generation of doubled haploids

Keeping in view the potential of the winter wheat ecotype, certain elite genotypes of this group were crossed to introgress genes for drought and cold tolerance, highly fertile square heads, profuse tillering and resistance to various biotic stresses into the spring wheat ecotype. Accordingly, 21 winter × spring wheat-derived F_1s were generated, which were further subjected to doubled haploid production through the chromosome elimination-mediated approach following a wheat × maize system for the rapid development of homozygous lines. Overall, on average, 311 florets were pollinated in each of the 21 elite and diverse winter × spring wheat F_1 hybrids with freshly collected maize pollen (grown in a polyhouse). From each cross, a variable frequency of pseudo-seeds, embryos, haploid regenerant and doubled haploid plants were obtained. Amongst all the crosses, Saptdhara × HPW 184 had the highest frequency of pseudo-seeds, higher embryo formation, and average regeneration but higher doubled haploid production. Saptdhara × HW 3024 had the highest frequency of doubled haploid lines, but low pseudo-seed formation and embryo formation and the highest regeneration. None of the cross combinations could attain high frequency for all the haploid induction parameters, which indicated that the genes controlling these parameters express themselves at different stages and are independently inherited.

Screening for drought tolerance

In vitro *screening for drought tolerance*

Analysis of variance for various laboratory characters in control and stress environments indicated sufficient genotypic differences and the differential response of the genotypes to different stress levels for all the parameters. Regarding the variability parameters of the *in vitro* characters (Table 1), the mean values were generally higher under control (E_1) conditions than in the stress environment (E_2) for all the traits except the root/shoot ratio. The mean root/shoot ratio was higher under stress than in the control environment, which indicated that the shoot length was drastically affected by the stress environment, in agreement with the results of Dhanda et al. (2004). The possible cause of the increased root/shoot ratio under water stress may be the limited water supply and nutrients to the shoot and some hormonal message induced in the root when they encounter water stress (Sharp and Davis, 1989). The range of mean values was greater under stress conditions in comparison to the control, indicating the low expression of the characters by certain genotypes under stress conditions. The greatest reduction in the mean values under stress (E_2) as compared to the control (E_1) environment was observed for shoot length (92.62%), followed by seedling vigour index (89.13%), root length (83.23%) and coleoptile length (65.52%). This indicated greater sensitivity to water stress at the seedling stage. In conformity to the present results, Dhanda et al. (2004) also observed that seed vigour index was the most sensitive trait, followed by shoot length, germination percentage and root length.

Table 1
Mean, range and variability parameters in control/irrigated (E_1) and stress/rainfed (E_2) environments for various *in vitro* and *in vivo* parameters

Traits	1	2	3	4	5	6	7	8
In vitro parameters								
Germination (%)	E_1	78.7–100.0	96.1		5.23	3.61	47.80	5.14
	E_2	13.3–95.3	77.9	18.94	22.18	21.69	95.70	43.72
Shoot length (cm)	E_1	7.2–19.4	12.2		20.01	14.61	53.30	21.97
	E_2	0.2–3.9	0.9	92.62	104.61	91.10	75.80	166.67
Root length (cm)	E_1	7.9–22.8	16.1		25.11	20.92	69.40	35.84
	E_2	0.2–8.2	2.7	83.23	60.33	52.54	75.80	95.19
Root/shoot ratio	E_1	0.8–3.1	1.4		32.04	21.85	46.50	30.00
	E_2	1.1–12.1	4.4	–2.14	59.38	41.66	49.20	59.77
Root number	E_1	3.0–5.7	4.2		21.30	14.23	44.60	19.52
	E_2	1.0–5.3	3.2	23.81	31.28	23.18	54.90	35.31
Coleoptile length (cm)	E_1	1.7–4.8	2.9		19.81	17.22	75.50	30.69
	E_2	0.2–3.4	1.0	65.52	86.43	76.14	77.60	134.00
Seedling vigour index	E_1	1444.07–4040.0	2719.0		21.16	17.65	69.60	30.33
(%)	E_2	21.7–877.9	295.6	89.13	73.31	66.69	82.80	124.97
In vivo parameters								
Days to flowering	E_1	112.7–182.3	137.6		9.64	9.54	98.0	19.46
	E_2	107.3–172.0	133.2	3.20	9.40	9.34	98.7	19.11
Days to maturity	E_1	153.7–212.3	172.3		7.31	7.08	93.9	14.14
	E_2	145.7–205.7	163.6	5.05	8.28	8.17	97.2	16.59
Plant height (cm)	E_1	54.1–105.8	75.8		15.38	14.34	86.9	27.53
	E_2	45.2–92.9	70.1	7.52	17.54	15.69	80.0	28.92
Flag-leaf area (cm^2)	E_1	3.7–38.5	12.7		47.64	44.79	88.4	86.69
	E_2	3.7–38.3	10.8	14.96	51.80	48.66	88.3	93.80
Tillers/plant	E_1	1.5–5.3	3.2		26.53	14.60	30.3	16.56
	E_2	1.2–5.1	2.6	18.75	30.41	23.32	58.8	36.92
Spike length (cm)	E_1	7.1–13.4	10.1		13.87	12.45	80.6	23.17
	E_2	6.7–13.2	9.8	2.97	12.06	10.51	76.0	18.88
Spikelets/spike	E_1	15.5–33.7	19.6		15.86	10.11	40.6	13.27
	E_2	11.5–25.1	17.7	9.69	14.79	11.23	57.7	17.57
Grains/spike	E_1	28.4–68.3	45.4		19.33	13.01	45.3	18.02
	E_2	9.9–54.7	37.0	18.50	27.95	18.31	42.9	24.70
Grain yield/plant (g)	E_1	1.820–7.017	4.206		28.71	24.10	70.5	41.61
	E_2	0.845–6.083	3.241	22.94	34.78	29.04	69.7	49.98
Grain yield weight (g)	E_1	33.57–55.36	42.31		12.13	10.02	68.2	17.04
	E_2	27.60–53.23	38.38	9.29	14.66	11.86	65.4	19.75
HI (%)	E_1	27.17–66.45	42.65		16.01	12.56	61.6	20.30
	E_2	12.09–48.07	36.63	14.12	21.06	15.14	51.7	22.41
RWC (%)	E_1	65.12–98.01	85.23		7.09	5.61	62.6	9.15
	E_2	54.53–95.55	80.41	5.66	9.00	7.70	73.3	13.58
ELWL (T%)	E_1	54.35–97.45	71.06		19.03	14.90	61.2	24.02
	E_2	27.58–96.86	59.32	16.52	23.63	18.95	64.3	31.30

Note: 1: environments; 2: range; 3: mean; 4: % decrease in mean in E_2; 5: Phenotypic coefficient of variation (PCV %); 6: Genotypic coefficient of variation (GCV %); 7: h^2_{bs} (%); 8: Genetic advance (%); HI: Harvest index; RWC: Relative water content; ELWL: Excised leaf water loss;

High phenotypic and genotypic coefficients of variation were observed for shoot length, root length, coleoptile length and seedling vigour index in the stress environment (data not given). Based upon the estimates of heritability and genetic advance, high genetic gains can be expected by exercising selection for shoot length, root length, coleoptile length and seedling vigour index under stress conditions.

Amongst the top one-third better performing genotypes (data not given), the parental genotypes Saptdhara, Sentry and VWFW 499 and the DH lines DH 63, DH 80, DH 85 and DH 69 performed significantly better for most of the traits under control (0 MPa) conditions, whereas under stress (–0.7 MPa) conditions, the parental genotype VWFW 499 and the doubled haploids DH 19, DH 61, DH 53, DH 26, DH 20, DH 55 and DH 36 were better for most of the traits and DH 28 performed significantly better with respect to all the traits.

In vivo *screening for drought tolerance*

Under rainfed conditions the crop suffered from water stress during the crown root initiation, late jointing, flowering and grain-filling periods (Table 2). Analysis of variance for various *in vivo* parameters in the irrigated and rainfed environments indicated enormous genetic variability as well as the differential response of the genotypes to these two environments for all the parameters. All the traits were significantly affected in the rainfed environment (Table 1). However, the maximum reduction as a result of the stress was observed for grain yield per plant (22.94%), followed by tillers per plant (18.75%), grains per spike (18.50%), excised leaf water loss (16.52%), flag-leaf area (14.96%) and harvest index (14.12%). Amongst the various traits studied, spikelets per spike, 1000-grain weight, plant height, relative water content, days to maturity, days to flowering and spike length were only marginally affected, but water stress adversely affected the grain yield per plant, tillers per plant and grains per spike, which is in accordance with the findings of Dencic et al. (2000). Considering the magnitude of heritability and genetic advance for various plant traits, selection for flag-leaf area, tillers per plant and excised leaf water loss may be effective when breeding for drought tolerance.

The top one-third genotypes were identified on the basis of their performance with respect to each trait under irrigated (E_1) and rainfed (E_2) conditions (data not given). All the genotypes behaved differently with respect to all these traits. On the basis of overall ranking, none of the genotypes performed better with respect to all the traits simultaneously under irrigated and rainfed conditions. Under irrigated conditions, the parental genotypes, Saptdhara and Sentry, and the DH 77 line performed better for most of the traits. Likewise, the parental genotypes, Saptdhara, Sentry and VWFW 452 performed better in a rainfed environment. Almost all the DH lines amongst the top one-third genotypes under both environments were found to be significantly better for only two or three traits, while having average performance for the rest of the parameters studied.

Table 2
Distribution of rainfall, temperature regimes and drought stress during the crop season (2003–04)

Period	Temperature (°C)		Rainfall (mm)	Stress (No. of days)	Crop growth stage
	Max.	Min.			
Nov. 20–Dec. 14	20.5	7.3	4.3	25	Germination
Dec. 15–Jan. 11	16.9	5.4	11.4	28	CRI* and tillering
Jan. 12–Feb. 8	15.8	4.8	246.9	28	Tillering
Feb. 9–March 8	21.6	9.1	35.9	29	Jointing
March 9–April 12	28.0	14.1	2.1	35	Anthesis
April 13–May 12	28.8	16.2	80.9	30	Grain filling and dough
May 13–June 5	32.9	19.4	48.6	24	Dough and maturity
Total			430.1	199	

*: CRI: crown root initiation

Relative drought tolerance based on drought susceptibility index

Drought susceptibility index (DSI) values were calculated for all the derivatives (Table 3) and ranged from –0.03 to 3.70 with a moderate drought intensity (D) of 0.23. Since a lower value of DSI indicates a greater degree of drought tolerance, DH 4 was found to be the most drought-tolerant genotype, because it exhibited the lowest DSI value (–0.03), followed by DH 8 (0.03), Saptdhara (0.05), DH 23 (0.08) and other doubled haploids. DH 4 had an index value less than zero (–0.03), which shows the genetic potential of this line to give higher yield in a rainfed environment, making it specifically suited to be grown under rainfed conditions, for which this line has specific adaptability.

Amongst these drought-tolerant genotypes, only a few genotypes (the winter wheat parents, Saptdhara, VWFW 452, Sentry and W 10 and the DH lines DH 6, DH 20 and DH 93) were found to be superior to the check, HPW 155, and were thus identified as potential genotypes on the basis of yield performance as well as the drought susceptibility index.

Correlation studies

The correlation coefficients calculated between various *in vitro* parameters (Table 4) revealed the positive association of germination percentage with shoot length, root length, coleoptile length and seedling vigour index under stress conditions. This is because moisture stress stimulated a significant and progressive reduction in germination percentage, shoot length and root length. The relationship between germination percentage and the seedling vigour index is due to the fact that germination percentage is a component of the index and has a direct bearing on the latter. The seedling vigour index was correlated with all the characters in both the environments, except the root number in the control and the root/shoot ratio under stress conditions, indicating that it can be used as a criterion for screening the genotypes for drought tolerance. The correlation coefficients of 'S' exhibited a significant negative association with germination percentage, root number and coleoptile length, indicating that these parameters may be selected for drought tolerance, as genotypes having a high germination percentage coupled with a higher number of roots and longer coleoptiles would tend to lower the value of 'S' in a stress environment, and a lower value of 'S' indicates greater drought tolerance.

Table 3
Drought susceptibility index (S) of the genotypes

Genotype	DSI	Genotype	DSI	Genotype	DSI	Genotype	DSI
DH 1	1.11 S	DH 31	0.85 I	DH 60	1.30 S	DH 88	0.62 T
DH 3	1.26 S	DH 32	1.41 S	DH 61	1.38 S	DH 89	0.92 I
DH 4	–0.03 T	DH 33	0.96 I	DH 62	1.92 S	DH 93	0.25 T
DH 5	0.44 T	DH 34	0.39 T	DH 63	1.31 S	HPW 42	0.82 I
DH 6	0.37 T	DH 35	2.12 S	DH 64	0.48 T	HPW 89	1.43 S
DH 7	1.55 S	DH 36	0.37 T	DH 65	0.80 I	HPW 147	0.81 I
DH 8	0.03 T	DH 38	1.23 S	DH 66	0.92 I	HPW 155	0.17 T
DH 10	1.57 S	DH 39	0.78 I	DH 67	0.22 T	HPW 184	0.91 I
DH 11	0.10 T	DH 40	0.44 T	DH 68	0.30 T	HW 3024	1.26 S
DH 14	0.76 I	DH 41	0.40 T	DH 69	1.87 S	PBW 343	0.83 I
DH 15	1.17 S	DH 43	1.16 S	DH 70	1.01 S	PW 552	1.01 S
DH 16	1.27 S	DH 44	1.12 S	DH 71	0.79 I	UP 2418	1.49 S
DH 17	0.27 T	DH 45	0.61 T	DH 72	1.39 S	Saptdhara	0.05 T
DH 18	0.36 T	DH 46	1.55 S	DH 73	1.10 S	Sentry	0.27 T
DH 19	2.75 S	DH 47	1.09 S	DH 74	0.35 T	VWFW 452	0.13 T
DH 20	0.29 T	DH 48	0.20 T	DH 75	0.22 T	VWFW 499	0.21 T
DH 21	0.15 T	DH 49	0.29 T	DH 76	1.84 S	W 10	0.51 T
DH 22	3.70 S	DH 51	1.55 S	DH 77	2.92 S	WW 24	1.79 S
DH 23	0.08 T	DH 52	0.31 T	DH 78	1.70 S		
DH 25	2.17 S	DH 53	0.64 T	DH 79	2.28 S		
DH 26	1.96 S	DH 55	1.13 S	DH 80	0.41 T		
DH 27	0.82 I	DH 56	1.07 S	DH 84	2.40 S		
DH 28	0.48 T	DH 57	0.20 T	DH 85	0.56 T		
DH 29	1.87 S	DH 58	0.97 I	DH 86	0.38 T		
DH 30	0.88 I	DH 59	0.82 I	DH 87	0.37 T		

Note: DSI: drought susceptibility index; T: Drought-tolerant; I: Intermediate; S: Drought-susceptible

Table 4
Correlation coefficients between various *in vitro* parameters and the drought susceptibility index (S) in control (E_1) and stress (E_2) environments

Traits		1	2	3	4	5	6	S
Germination (%)	E_1	0.008	0.083	–0.007	–0.046	0.001	0.298*	–0.15
	E_2	0.257*	0.239*	–0.086	0.074	0.205*	0.471*	–0.21*
1	E_1		0.512*	–0.063	0.003	0.546*	0.754*	–0.16
	E_2		0.594*	–0.499*	0.282*	0.830*	0.825*	–0.18
2	E_1			0.449*	–0.188	0.412*	0.910*	0.04
	E_2			0.085	0.280*	0.621*	0.899*	–0.10
3	E_1				–0.092	0.001	0.276*	0.11
	E_2				–0.077	–0.509*	–0.167	0.10
4	E_1					–0.143	–0.139	–0.13
	E_2					0.292*	0.290*	–0.39*
5	E_1						0.507*	–0.02
	E_2						0.751*	–0.21*
6	E_1							–0.06
	E_2							–0.15

1: Shoot length (cm); 2: Root length (cm); 3: Root/shoot ratio; 4: Root number; 5: Coleoptile length (cm); 6: Seedling vigour index (%); *Significant at $P \leq 0.05$.

Correlation studies on various *in vivo* parameters (Table 5) showed that the magnitude of the correlation coefficients for most of the plant traits with grain yield per plant was higher in the irrigated (E_1) environment as compared to the rainfed (E_2) environment. Grain yield per plant showed a positive association with spike length, grains per spike, 1000-grain weight and harvest index in both the environments. Under rainfed conditions, this trait was positively correlated with flag-leaf area, spikelets per spike and relative water content and negatively associated with excised leaf water loss. Tillers per plant was positively correlated with grain yield per plant in the case of irrigation and with spikelets per spike, grains per spike, 1000-grain weight and harvest index in the rainfed environment. The significantly positive and negative associations of 'S' with grain yield in the irrigated and rainfed environments, respectively, indicated that sensitive genotypes had higher grain yield under adequate water supplies, while tolerant genotypes gave higher grain yield under stress conditions. The significantly negative association of tillers per plant and relative water content with 'S' signifies the importance of this trait as a selection criterion for drought tolerance in a rainfed environment, as genotypes with a higher number of tillers per plant and high relative water content would be drought-tolerant in a stress environment.

Rank correlation

The significant positive rank correlation between the *in vitro* and *in vivo* stress conditions (rs = 0.2403*) indicated that the performance of a genotype under field conditions was similar to its performance under laboratory conditions. Thus, *in vitro* screening parameters, especially germination percentage, root number, coleoptile length and seedling vigour index, may be efficiently used to screen the genotypes for drought tolerance. Under *in vivo* conditions, relative water content and excised leaf water loss were found to be the most useful parameters for screening for drought tolerance, as these characters are related with each other as well as with the drought-related trait 'S'.

The top one-third of genotypes with better performance in a rainfed environment were compared with the top one-third genotypes in the stress (–0.7 MPa) environment. Amongst these genotypes, only four winter wheat parents, Saptdhara, Sentry, VWFW 452 and VWFW 499, and nine DH lines, DH 1, DH 10, DH 20, DH 21, DH 26, DH 55, DH 57, DH 77 and DH 86, were identified as better performing genotypes in both the environments.

When the drought susceptibility index of these genotypes was also taken into consideration, all the four better performing parents (Saptdhara, Sentry, VWFW 452 and VWFW 499) and four DH lines (DH 20, DH 21, DH 57 and DH 86) were categorized as drought-tolerant, whereas the remaining five DH lines (DH 1, DH 10, DH 26, DH 55 and DH 77) were drought-susceptible (Table 3).

Table 5
Correlation coefficients between various *in vivo* parameters and the drought susceptibility index (S) in irrigated (E_1) and rainfed (E_2) environments

Traits	E	2	3	4	5	6	7	8	9	10	11	12	13	S
1	E_1	0.89*	–0.29*	–0.16	0.21*	0.33*	0.06	–0.22*	0.12	0.07	–0.22*	–0.17	–0.01	0.09
	E_2	0.87*	–0.28*	–0.26*	0.32*	0.14	0.29*	–0.14	0.04	–0.03	–0.26*	–0.24*	–0.06	0.10
2	E_1		–0.19	–0.01	0.13	0.39*	0.05	–0.20	0.14	0.16	–0.17	–0.15	–0.00	0.12
	E_2		–0.30*	–0.07	0.23*	0.17	0.19	–0.18	0.04	0.00	–0.30*	–0.21*	–0.04	0.10
3	E_1			0.00	–0.22*	0.15	0.10	0.02	–0.10	0.07	0.05	–0.00	–0.01	–0.14
	E_2			0.15	0.08	0.35*	0.10	0.29*	0.08	0.33*	0.18	0.04	–0.01	–0.16
4	E_1				–0.06	0.11	–0.08	0.08	0.15	0.32*	0.14	0.38*	–0.10	–0.08
	E_2				–0.10	0.06	–0.03	0.17	0.32*	0.33*	0.25*	0.30*	–0.20	–0.16
5	E_1					0.09	0.08	0.14	0.33*	0.16	0.17	0.03	0.03	–0.16
	E_2					0.16	0.32*	0.32*	0.19	0.29*	0.21*	–0.03	–0.11	–0.27*
6	E_1						0.13	0.01	0.26*	0.29*	0.03	–0.08	0.03	0.17
	E_2						0.34	0.10	0.21*	0.24*	–0.05	–0.19	–0.06	–0.11
7	E_1							0.11	0.09	0.15	–0.02	–0.06	–0.09	–0.07
	E_2							0.39*	0.27*	0.17	0.23*	0.07	–0.18	–0.08
8	E_1								0.32*	0.05	0.21*	0.19	–0.01	–0.02
	E_2								0.29*	0.31*	0.63*	0.30*	–0.10	–0.06
9	E_1									0.41*	0.35*	0.06	–0.01	0.27*
	E_2									0.41*	0.29*	0.24*	–0.26*	–0.59*
10	E_1										0.24*	0.02	0.12	–0.04
	E_2										0.34*	0.14	–0.13	–0.16
11	E_1											0.17	0.04	0.07
	E_2											0.39*	–0.16	–0.16
12	E_1												–0.17	–0.20
	E_2												–0.23*	–0.22*
13	E_1													0.12
	E_2													0.19

1: Days to flowering; 2: Days to maturity; 3: Plant height (cm); 4: Flag-leaf area (cm^2); 5: Tillers/plant; 6: Spike length (cm); 7: Spikelets/spike; 8: Grains/spike; 9: Grain yield/plant (g); 10: 1000-grain weight(g); 11: Harvest index (%); 12: Relative water content (%); 13: Excised leaf water loss (%) *Significant at $P \leq 0.05$

Thus, categorizing the genotypes as tolerant or susceptible on the basis of the drought susceptibility index 'S' alone is not a reliable criterion, as it is based on the minimization of yield reduction under stress as compared with the non-stress environment and does not differentiate between potentially drought-tolerant genotypes and those that had low yield potential from other causes. This is in agreement with the results of Clarke (1992). Hence, the drought susceptibility index along with other traits that confer drought tolerance and are expressed better in a stress environment, such as the *in vitro* parameters germination percentage, root number, coleoptile length and seedling vigour index and the *in vivo* parameters relative water content and excised leaf water loss, should be taken into consideration when categorizing genotypes as tolerant or susceptible, so as to enhance the selection precision for the crucial and complex trait of drought.

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HETEROSIS, INBREEDING DEPRESSION AND THEIR RELATIONSHIP WITH GENETIC DIVERGENCE IN SESAME (*Sesamum indicum* L.)

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Seven parents (CST2002, MT34, OS-Sel-2, TKG22, AAUDT9304-14-4, B67 and Rama), their 21 F_1s and 21 F_2s were grown in summer 2003 in a randomized block design with three replications. Heterosis and inbreeding depression were studied for seven important yield-contributing characters (plant height, branch number plant^{-1}, capsules plant^{-1}, seeds capsule^{-1}, 1000-seed weight, stick yield plant^{-1} and seed yield plant^{-1}). Maximum heterosis for seed yield plant^{-1} over the mid- and better-parent was recorded in CST2002×TKG22 (43.30%) and MT34×B67 (27.22%), respectively. Mid-parent heterosis for seed yield plant^{-1} was due to cumulative heterosis for various important component traits, such as capsules plant^{-1}, seeds capsule^{-1} and 1000-seed weight. Inbreeding depression was highest for seed yield, followed by 1000-seed weight, capsules plant^{-1}, branch number and plant height, indicating the predominance of non-additive genetic effects. B67×Rama exhibited significant positive heterosis in F_1, but non-significant inbreeding depression in F_2 for seed yield. This cross can be utilized as basic material for identifying better pure lines. The clustering pattern indicated that in general genetically diverse parents exhibited more heterosis, as evident in the majority of the crosses.

Key words: sesame, *Sesamum indicum,* morphological characters, heterosis, inbreeding depression, genetic divergence

Introduction

Sesame (*Sesamum indicum* L.) is one of the most important ancient oilseed crops, grown throughout the tropical and sub-tropical regions of the world (Ashri, 1998). Globally about 3,321,458 tons of sesame are produced on 7,554,200 ha (Uzun et al., 2007). India, Sudan, Myanmar and China are the most important sesame producers, with 68% of the total world production (Laurentin and Karlovsky, 2006). Sesame contains about 50–60% seed oil (Uzun et al., 2002), which is of superior quality. Sesame oil is highly stable (Uzun et al., 2007) compared to other edible oils, mainly due to the presence of antioxidants like sesamin, sesaminol, sesamol, sesamolinol and squalene

(Mohamed and Awatif, 1998). Sesame oil has a reducing effect on the plasma cholesterol and, in conjunction with blood pressure-lowering medicine, it also lowers the blood pressure (Sankar et al., 2005). Despite its importance as an oilseed crop, research on sesame has been scarce (Bedigian, 2003). The potential benefits of sesame for human health have recently led to renewed interest in this ancient crop (Laurentin and Karlovsky, 2006). The average productivity of this important oil seed crop in India is only 453 kg ha^{-1}, which is far below the average productivity in China (1127 kg ha^{-1}) and Egypt (1211 kg ha^{-1}) (Banerjee and Kole, 2009). One of the most promising approaches to raise the productivity of sesame may be the exploitation of heterosis.

Heterosis and inbreeding depression are considered two aspects of the same phenomenon (Falconer, 1981). In contrast to heterosis, inbreeding depression refers to the reduced fitness of progenies resulting from inbreeding (Stuber, 1994). Therefore, it is desirable to study inbreeding depression along with heterosis to distinguish whether the observed vigour in F_1 breaks down in F_2 or not.

In the present investigation, heterosis was estimated as the difference between the hybrid and the mean of the two parents (mid-parent heterosis; Falconer and Mackay, 1996) and the hybrid and the better parent (better-parent heterosis), the latter being preferred in many circumstances.

This investigation was conducted to study heterosis and inbreeding depression and to identify crosses for the development of hybrids and/or potential source materials to isolate superior segregants. It has been established from studies on several crops that allelic divergence plays an important role in the manifestation of heterosis (Moll et al., 1962), though it does not necessarily follow that this relationship will hold throughout the entire range of diversity in the species (Moll et al., 1965). An attempt was also made to discover the possible role of the genetic divergence present among the parents in the expression of heterosis in sesame hybrids with respect to seed yield and important components thereof.

Materials and methods

Seven morphologically diverse sesame genotypes, representing the major sesame growing regions of India, namely CST2002 and MT34 from north-central India, OS-Sel-2 from south-eastern India, TKG22 from central India, AAUDT9304-14-4 from north-eastern India, B67 and Rama from eastern India, were crossed in a half-diallel mating system during summer (March–June) 2002 to produce 21 F_1 hybrids. Among the selected parents TKG22 (Duhoon et al., 2004), Rama and B67 were used as national and zonal checks in various breeding programmes in India. In the post-rainy season (August–November) of 2002, a small portion of the F_1 seed produced in summer 2002 was sown (2 rows each) and allowed to self-pollinate to produce 21 F_2s. The entire selfing block (42 rows) was surrounded from all sides with insect-proof net to avoid any kind of out-crossing through insect pollination. All the seven parents, 21 F_1s and 21 F_2s were grown in a randomized complete block design with three replications during summer (March–June) 2003 in the Agricultural Farm, Institute of Agriculture, Visva-Bharati University, Sriniketan (23°39′N, 87°42′E and 58.9 masl), located in the sub-humid, sub-tropical belt of West Bengal, India. The

experimental plot was sandy loam in texture (61% sand, 10.7% silt and 28.3% clay) and acidic in nature (pH 5.2) with total available N: 235.4 kg ha^{-1}, P: 20.4 kg ha^{-1}, K: 172.3 kg ha^{-1} and organic C: 0.5%. All the parents and F_1s were grown in 5 rows 3 m in length, while all the F_2s were grown in 10 rows 3 m in length. A uniform spacing of 30 cm between rows and 15 cm between plants was followed. Sowing was done manually in optimum moisture conditions in furrows by placing 2 to 3 seeds per hill (at 15 cm intervals). Thinning was done 20 days after sowing to maintain a proper plant stand. The standard agronomic package of practices was followed and suitable plant protection measures were taken to raise a healthy crop.

Observations were recorded on plant height in cm (PH), branch number $plant^{-1}$ (BN), capsules $plant^{-1}$ (CP), seeds $capsule^{-1}$ (SC), 1000-seed weight in g (TW), stick yield $plant^{-1}$ in g (STY) and seed yield $plant^{-1}$ in g (SY) from 10 randomly selected plants from each plot for the parents and F_1s and from 30 plants from each plot for the F_2s in each replication. Genetic dissimilarity between the seven parents was estimated based on the above-mentioned seven characters and additionally for days to initiation of flowering (DIF), days to complete flowering (DCF) and days to maturity (DM) (data not presented). Since the parents were indeterminate in growth habit, data were recorded for days to initial and complete flowering rather than days to 50% flowering.

The magnitude of heterosis was estimated for all seven traits and expressed as the percentage superiority or inferiority of the F_1 hybrids over the mid-parent (H_{mp}, relative heterosis) and the better parent (H_{bp}, heterobeltiosis). Inbreeding depression (ID) was computed as the percentage superiority or inferiority of the F_1 hybrids over the F_2 mean.

$$H_{mp} = (F_1 - MP)/MP \times 100;\ H_{bp} = (F_1 - BP)/BP \times 100;\ ID = (F_1 - F_2)/F_1 \times 100$$

where, F_1 = F_1 mean; F_2 = F_2 mean; BP = better-parent mean; MP: mid-parent mean.

For the estimation of genetic diversity among the seven parents each trait value was transformed to an index (I) by the following formula:

$$I_{ij} = T_i/GM$$
$$i = 1, 2 \ldots 7;\ j = 1, 2 \ldots 10$$

where, I_{ij} = index value for the 'i'th parent for the 'j'th trait; T_i = mean of all the F_1s involving the 'i'th parent; GM = general mean of all the F_1s.

Genetic dissimilarity between the parents was estimated from the Euclidean distance matrix estimated from the index value for each trait. The Euclidean distance was estimated by the following formula:

$$D_{rs} = [\sum(X_{rj} - X_{sj})^2]^{1/2}$$

where X_{rj} and X_{sj} are the 'r'th and 's'th objects measured on the 'j'th variable.

Once the distance matrix was estimated, a dendogram was constructed based on the average linkage method known as Unweighted Pair Group Method using the Arithmetic mean (UPGMA) (Sneath and Sokal, 1973). Hierarchical clustering was performed using R-Statistical software (using 1000-boot strap sampling) and the Agricolae package.

Results

The relative heterosis (H_{mp}) and heterobeltiosis (H_{bp}) for the seven traits are presented in Table 1. Among the 21 F_1 hybrids, eight hybrids exhibited significant positive relative heterosis for PH, whereas only one of them, AAUDT9304-14-4×Rama, significantly exceeded the better parent (6.96%). Five crosses exhibited significant negative relative heterosis for PH, while seven of them showed significant negative heterobeltiosis for this trait. Three crosses showed significant positive relative heterosis for BN, though only one of them, OS-Sel-2×B67, showed heterobeltiosis (70.13 and 35.05%, relative heterosis and

heterobeltiosis, respectively). Ten F_1 hybrids exhibited significant positive relative heterosis for CP, of which four: OS-Sel-2×B67 (37.38%), OS-Sel-2×Rama (24.57%), AAUDT9304-14-4×Rama (20.78%) and TKG22×Rama (16.73%), exhibited significant positive heterobeltiosis for this trait. The number of hybrids exhibiting significant positive heterosis was less for SC than for the other characters. OS-Sel-2×Rama showed highly significant positive relative heterosis and heterobeltiosis (45.34% and 35.81%, respectively) for SC. For TW, OS-Sel-2×TKG22 (15.49%) and CST2002×MT34 (7.31%) displayed the highest values of significant positive relative heterosis and heterobeltiosis, respectively. Five hybrids showed significant positive relative heterosis for STY, while three of them exhibited the same trend over the better parent.

Maximum significant positive relative heterosis and heterobeltiosis for SY was shown by CST2002×TKG22 (43.30%) and MT34×B67 (27.22%), respectively. Other hybrids, namely TKG22×B67 (42.38 and 19.44%), TKG22×Rama (40.14 and 19.77%), MT34×B67 (38.79 and 27.22%), MT34×Rama (26.09 and 18.02%), OS-Sel-2×Rama (25.91 and 13.55%), CST2002×MT34 (25.50 and 10.05%), B67×Rama (24.43 and 21.67%), AAUDT9304-14-4×B67 (23.34 and 10.57%) and OS-Sel-2×AAUDT9304-14-4 (12.93 and 9.69%), had significant positive relative heterosis and heterobeltiosis for SY.

In the present investigation, inbreeding depression was evident in F_2 for most of the traits studied (Table 2). Significant positive inbreeding depression for PH, BN, CP, SC, TW, STY and SY was shown by 7, 9, 10, 4, 12, 5 and 18 crosses, respectively. The highest inbreeding depression was observed for TW, CP, BN and PH. Some of the crosses exhibiting significant positive relative heterosis also showed significant negative or non-significant but low inbreeding depression (Table 3). Three crosses, CST2002×AAUDT9304-14-4, OS-Sel-2×B67 and B67×Rama, showed non-significant inbreeding depression not only for SY, but also for one or more important component characters, such as CP, SC or TW. OS-Sel-2×B67 exhibited non-significant (in fact, negative, –2.03%) mid-parent heterosis in F_1 and also negative (–8.81%) inbreeding depression for SY.

Four different clusters were formed at a cut-off distance of 25 distance units (Fig. 1). Cluster I (Rama, AAUDT9304-14-4 and TKG22) could be subdivided into two sub-clusters. Sub-cluster I comprised Rama and AAUDT9304-14-4 (from north-eastern India) and sub-cluster II was represented by TKG22 (from central India). Cluster II was constituted by OS-Sel-2 and B67 (from eastern India). Cluster III (MT34) and cluster IV (CST2002) were mono-genotypic clusters both representing north-central India. The distribution pattern of the seven parents in different clusters indicated that genetic diversity was related with geographical diversity in the present set of parental genotypes. Among the 14 F_1 hybrids exhibiting significant positive relative heterosis, 10 hybrids significantly exceeded the better parent and all these hybrids except TKG22×Rama were inter-cluster crosses.

Table 1
Relative heterosis and heterobeltiosis for seven characters in sesame

Crosses	PH		BN		CP		SC	
	H(mp)	H(bp)	H(mp)	H(bp)	H(mp)	H(bp)	H(mp)	H(bp)
CST2002 × MT34	15.09**	–2.66	–3.20	–13.82*	3.56	–0.38	–0.04	–16.72*
CST2002 × OS-Sel-2	2.00	–3.09	5.88	–15.62*	–5.12	–15.51*	–2.77	–17.90*
CST2002 × TKG22	8.41*	–6.37	–26.09**	–29.17**	10.53	2.07	–7.33	–15.00*
CST2002 × AAUDT9304-14-4	–5.79	–7.16*	–13.73*	–18.52**	–11.15	–26.46**	3.26	–12.40
CST2002 × B67	4.94	2.31	–7.63	–4.12	–4.33	–15.81*	–12.17	–24.20**
CST2002 × Rama	–25.44**	–26.32**	–17.65**	–22.22**	–1.98	–14.39*	5.04	–16.04*
MT34 × OS-Sel-2	18.24**	4.54	10.00	–19.51**	–16.96*	–23.38**	–9.62	–11.11
MT34 × TKG22	6.83	4.20	–5.21	–18.70**	36.96**	22.07	13.90	2.42
MT34 × AAUDT9304-14-4	–7.47	–22.67**	–22.08**	–26.83**	–13.27*	–25.87**	27.50**	24.72*
MT34 × B67	3.38	–10.68**	8.18	–3.25	14.19*	4.05	10.58	6.04
MT34 × Rama	15.97**	–0.94	–33.33**	–37.40**	10.75	0.13	26.19*	19.78
OS-Sel - 2 × TKG22	–18.88**	–26.67**	20.00*	–1.14	32.40**	10.00	3.05	–5.94
OS-Sel - 2 × AAUDT9304-14-4	–0.03	–6.33	5.45	–19.44**	–4.44	–12.12*	22.89*	22.21*
OS-Sel - 2 × B67	1.20	–1.44	70.13**	35.05**	39.36**	37.38**	8.05	5.29
OS-Sel - 2 × Rama	4.17	0.12	1.82	–22.22**	27.36**	24.57**	45.34**	35.81**
TKG22 × AAUDT9304-14-4	12.18**	–4.30	–25.51**	–32.41**	37.45**	6.88	11.82	2.58
TKG22 × B67	–6.83	–17.75**	7.03	2.06	21.02**	–0.54	3.24	–3.47
TKG22 × Rama	10.75**	–3.37	–25.51**	–32.41**	20.10**	16.73*	17.87	1.21
AAUDT9304-14-4 × B67	6.44*	2.30	12.20*	6.48	18.23**	10.10	8.22	6.04
AAUDT9304-14-4 × Rama	9.83**	6.96*	–4.63	–4.63	28.66**	20.78**	19.44	11.03
B67 × Rama	–9.23**	–10.45**	–9.27	–13.89*	5.34	4.43	6.36	–2.97
Range Min	–25.24	–26.67	–33.33	–37.40	–16.96	–26.46	–12.17	–24.20
Max	18.24	6.96	70.13	35.05	39.36	37.38	45.34	35.81

Crosses	TW		STY		SY	
	H(mp)	H(bp)	H(mp)	H(bp)	H(mp)	H(bp)
CST2002 × MT34	15.47**	7.31**	–8.4*	–13.04*	25.5**	10.05*
CST2002 × OS-Sel-2	0.01	–1.10	–8.67*	–17.28**	5.08	1.4
CST2002 × TKG22	3.11	5.38	30.87**	30.45**	43.3**	15.88**
CST2002 × AAUDT9304-14-4	–5.46	–7.95*	5.06	3.87	–10.8**	–16.3**
CST2002 × B67	–5.37	–6.36*	15.14**	5.48	2.37	–2.51
CST2002 × Rama	–6.15*	–6.83*	45.74**	32.58**	15.36**	7.54
MT34 × OS-Sel-2	5.38	–1.06	–8.12*	–12.57*	23.63**	5.14
MT34 × TKG22	8.29**	–7.02**	–2.89	–7.54	22.06**	10.67
MT34 × AAUDT9304-14-4	10.71**	0.38	0.62	–5.51	25.2**	3.96
MT34 × B67	5.12	–1.35	–0.17	–12.75*	38.79**	27.22**
MT34 × Rama	0.62	–7.12**	0.83	–12.46*	26.09**	18.02**
OS-Sel - 2 × TKG22	15.49**	4.93	–16.43**	–24.08**	10.12*	–13.55**
OS-Sel - 2 × AAUDT9304-14-4	7.22*	3.92	–14.16**	–23.04**	12.93**	9.69**
OS-Sel - 2 × B67	–2.47	–2.52	–6.56	–21.73**	–2.03	–9.81*
OS-Sel - 2 × Rama	0.95	–0.88	–11.64**	–26.44**	25.91**	13.55**
TKG22 × AAUDT9304-14-4	12.44**	5.79	1.79	0.32	–11.17**	–31.72**
TKG22 × B67	14.84**	4.39	7.02	–2.24	42.38**	19.44**
TKG22 × Rama	14.76**	6.02	1.41	–8.01	40.14**	19.77**
AAUDT9304-14-4 × B67	–1.02	–4.61	5.17	–2.64	23.34**	10.57**
AAUDT9304-14-4 × Rama	3.36	1.36	18.49**	8.91	–16.29**	–26.43**
B67 × Rama	–11.5**	–13.05**	35.55**	34.5**	24.43**	21.67**
Range Min	–11.50	–13.05	–16.43	–26.44	–16.29	–31.72
Max	15.49	7.31	45.74	34.5	43.3	27.22

*, **: Significant at P = 0.05 and 0.01, respectively. H (mp): relative heterosis; H (bp): heterobeltiosis; PH: plant height (cm); BN: branch number plant^{-1}; CP: capsules plant^{-1}; SC: seeds capsule^{-1}; TW: 1000-seed weight (g); STY: Stick yield plant^{-1} (g); SY: seed yield plant^{-1} (g)

Table 2
Inbreeding depression in the F_2 generation for seven characters in sesame

Crosses	PH	BN	CP	SC	TW	STY	SY
CST2002×MT34	21.70**	1.89	11.21	8.31	24.64**	–14.67	18.26**
CST2002×OS-Sel-2	10.12*	39.51**	1.97	10.14	0.00	–6.96	18.43**
CST2002×TKG22	7.39	–36.76**	17.13	–0.32	5.68	19.16**	10.43**
CST2002×AAUDT9304-14-4	–2.00	–21.59**	–8.51	5.27	3.04	–28.88**	9.47
CST2002×B67	6.84	–4.30	10.22	6.54	1.76	28.13**	17.53**
CST2002×Rama	–32.78**	–15.48*	–1.45	7.35	1.44	–10.71	18.22**
MT34×OS-Sel-2	17.73**	18.18**	–2.60	14.20	10.01**	–16.47	13.33**
MT34×TKG22	5.32	18.00**	46.52**	31.94**	14.89**	–0.63	26.51**
MT34×AAUDT9304-14-4	–8.00	–21.11**	–6.45	7.56	23.18**	13.80	13.14**
MT34×B67	16.71**	18.49**	21.65**	14.36	7.60*	0.00	16.16**
MT34×Rama	3.66	–35.06**	11.76	1.48	14.49**	0.66	24.14**
OS-Sel - 2×TKG22	–27.58**	20.69**	32.11**	7.21	20.56**	–0.69	21.08**
OS-Sel - 2×AAUDT9304-14-4	7.49	45.98**	21.66**	27.75**	10.18**	–13.27	30.52**
OS-Sel - 2×B67	–2.41	35.11**	31.03**	13.18	1.57	2.34	–8.81
OS-Sel - 2×Rama	3.77	–26.19**	22.81**	23.49**	–0.22	–12.46	17.70**
TKG22×AAUDT9304-14-4	3.10	–30.14**	29.37**	12.60	12.40**	21.73**	16.77*
TKG22×B67	–11.41*	20.20**	29.89**	15.94	17.96**	–32.46**	23.26**
TKG22×Rama	24.72**	–34.35**	16.48	10.57	11.58**	–9.41	16.02**
AAUDT9304-14-4×B67	8.90*	13.04*	20.43**	16.59	3.68	–22.71**	15.54**
AAUDT9304-14-4×Rama	14.97**	6.80	30.94**	26.84**	7.96*	30.30**	34.73**
B67×Rama	–6.90	–3.23	9.20	–13.64	–10.21**	19.60**	7.76
Range Min	–32.78	–36.76	–8.51	–13.64	–10.21	–32.46	–8.81
Max	24.72	45.98	46.52	31.94	24.64	30.30	34.73
Mean	2.92	0.46	16.45	11.78	8.68	–1.60	17.15
No. of crosses with significant positive inbreeding depression	7	9	10	4	12	5	18

*, **: Significant at P = 0.05 and 0.01, respectively, PH: plant height; BN: branch number plant^{-1}; CP: capsules plant^{-1}; SC: seeds capsule^{-1}; TW: 1000-seed weight; STY: stick yield plant^{-1} (g); SY: seed yield plant^{-1}

Table 3
Sesame crosses with significant positive F_1 relative heterosis and significant negative or non-significant low inbreeding depression in F_2

Characters	Crosses
PH	TKG22×AAUDT9304-14-4 and MT34×Rama
BN	None
CP	TKG22×Rama
SC	MT34×Rama and MT34×AAUDT9304-14-4
TW	None
STY	CST2002×Rama
SY	B67×Rama

PH: plant height; BN: branch number plant^{-1}; CP: capsules plant^{-1}; SC: seeds capsule^{-1}; TW: 1000-seed weight; STY: stick yield plant^{-1}; SY: seed yield plant^{-1}

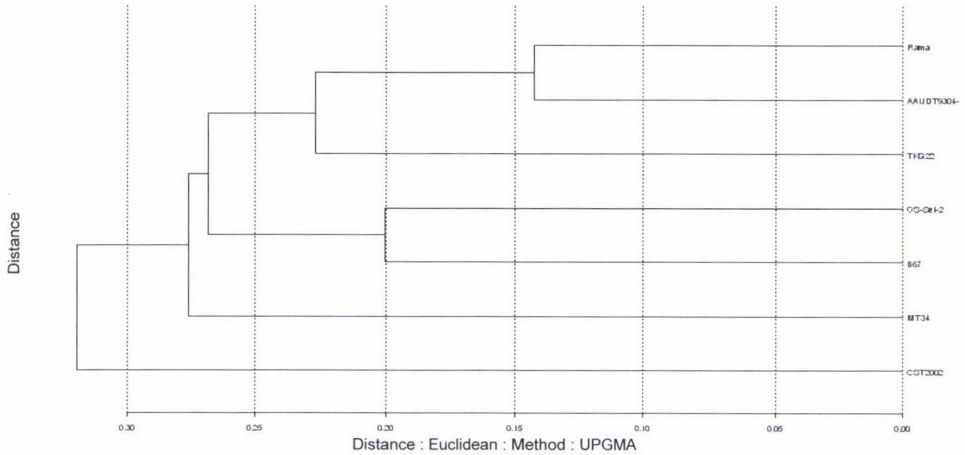

Fig. 1. Hierarchical clustering of seven parental genotypes

Discussion

Both positive and negative heterosis was observed for all the yield components studied. Analysis of the heterosis in the five best F_1 hybrids revealed that the relative heterosis for SY was due to cumulative heterosis for important component traits, like CP, SC and TW. Banerjee and Kole (2006a) reported the significant positive association of PH, BN, CP and SC with the seed yield in sesame. The performance of these hybrids is likely to be stable, as heterosis for yield is associated with heterosis for various yield components. However, the influence of other characters, apart from the morphological yield components studied, may have a role in the manifestation of heterosis in the seed yield of sesame. Banerjee and Kole (2006b) reported significant associations between physiological characters and the sesame oil yield. It is worth mentioning that negative heterosis for PH may be important for sesame breeders as it reduces the risk of plant lodging in the hybrids. On the other hand, positive heterosis, despite increasing the risk of lodging, may simultaneously increase the seed yield.

The presence of inbreeding depression in the F_2 generation indicated that non-additive effects were important for many of the crosses. Karuppaiyan and Ramasamy (2001) observed that, in general, crosses with significant heterosis also exhibited significant inbreeding depression in the F_2 generation. The increase in homozygosity in the F_2 generation reduces non-additive gene effects and reduces the mean phenotypic value. In self-pollinated species like sesame, the genetic load should be minimal. Natural selection and/or plant breeding in any form would be expected to eliminate deleterious gene mutations with large effects (Husband and Schemske, 1996). The heterosis observed for different traits in the present study might be due to the accumulation of a greater number

of dominant (favourable) alleles in the hybrids than in either of the pure-line parents. The genetic cause of non-additive variation can be fixed in pure-line cultivars. The existence of non-additive genetic effects might make early generation selection ineffective. However, early generation testing, when dominance is significant, would be useful if it could be done effectively and economically (Burton and Brownie, 2006). Bi-parental mating and reciprocal recurrent selection could also be effective.

The fact that SC and STY were comparatively less affected by inbreeding depression might be due to additive and additive × additive genetic effects, acting in the same favourable direction. Some of the crosses exhibiting significant positive heterosis also showed significant negative or non-significant but low inbreeding depression, possibly due to the preponderance of additive and additive × additive genetic effects. Most breeding programmes use pedigree selection or a modified form of this (Brim, 1966) and are designed to exploit both types of genetic effects. On the basis of heterosis, inbreeding depression and *per se* performance for SY and other component characters, B67 × Rama could be utilized for isolating high-yielding lines after the fixation of favourable additive genetic effects in advanced generations of segregation.

It cannot be concluded, at least from a theoretical point of view, that heterotic crosses will produce better pure lines than non-heterotic crosses. Yet the existence of heterosis could be evidence that superior gene combinations are possible, combined with a proper, effective selection scheme. It is worth mentioning that in the cross OS-Sel-2×B67, mean SY was higher in the F_2 generation than in F_1. The non-significant deviation of mean F_1 SY from the mid-parent might be due to the preponderance of additive genetic effects, though dominance cannot be completely ruled out. In sesame, yield being quantitative in nature, several dominant alleles acting in opposite directions might nullify the overall effect. Again, the higher F_2 mean SY might be due to the fixation of favourable alleles as homozygosity increases and is probably due to the presence of higher frequencies of transgressive segregants in the F_2 generation. This further confirmed the presence of a good amount of additive genetic components effective for SY in this particular cross.

The present findings supported the possible role of allelic divergence in the expression of the majority of the heterotic hybrids, with the exception of TKG22×Rama. In cluster I, TKG22 was comparatively distantly related to the other two genotypes (AAUDT9304-14-4 and Rama) and this might be the reason for heterotic expression in this cross. Ramanujam et al. (1974) reported that hybrids between genetically diverse parents were more heterotic. Melchinger (1999) reported a relationship between genetic diversity and heterosis in maize, although the correlation was not strong enough to be used as an accurate predictive tool. It may be suggested that genetic divergence in combination with other background information might be useful for the sesame breeder for the exploitation of heterosis and to obtain desirable segregants.

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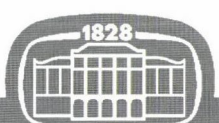

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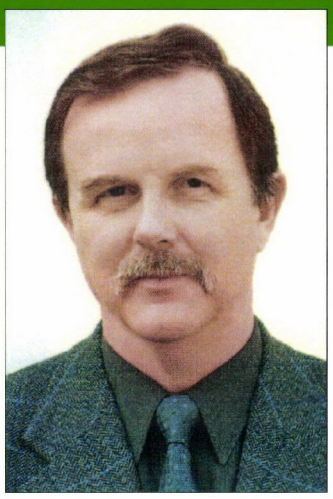

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REVIEWS

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COMBINED EFFECT OF THE DROUGHT DURATION AND ELEVATED ATMOSPHERIC CO_2 LEVEL ON PHYSIOLOGICAL AND YIELD PARAMETERS OF WINTER WHEAT

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The unfavourable effects of climate change were studied in terms of changes in the stress tolerance of cereals. The yield and physiological parameters of two winter wheat genotypes (Mv Mambó, Mv Regiment) were analysed in the phytotron after water was completely withheld for 7 or 14 days in three phenophases. The plants were raised in climate chambers, one adjusted to ambient CO_2 concentration and the other to a higher level (750 μmol mol^{-1}). The aim of the present work was to determine the correlations between the duration of water withholding and the phenological, physiological and yield parameters of winter wheat. It was hoped to identify how elevated CO_2 levels affected the stress sensitivity of plants and whether they contributed to counteracting the damaging effects of drought. In both varieties, the grain mass decreased to the greatest extent when water was withheld at first node appearance (5.9–71.3%). A longer period of drought at first node appearance and grain filling only reduced the grain number and mass in the case of enhanced CO_2. The yield and physiological parameters of Mv Regiment, however, deteriorated substantially as a result of water deficiency, though this variety was better able to utilise surplus CO_2, giving outstanding results at elevated CO_2 level.

Key words: drought stress, elevated CO_2, cereals, yield parameters, protein content, photosynthetic activity

Introduction

Agricultural production, including the cultivation of cereals, must adapt to the predicted changes in the global climate, some of which may be favourable, but many of which may lead to abiotic and biotic stress effects. Due to the very variable weather in the Carpathian Basin, crop producers may be faced with various challenges, even within a single vegetation period. In Eastern Central Europe the mean temperature rose during the second half of the 20th century, while the quantity of rainfall during the growing period decreased, thus increasing the level of risk for crop production (Bartholy and Pongrácz, 2007).

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The duration of drought periods also tends to be longer now, so water deficiency is likely to have an increasing influence on cereal yields (Brázdil et al., 2009).

The increasingly frequent occurrence of water deficiency due to climate change has a substantial effect on the development of cereal plants and thus on the yield (Barnabás et al., 2008; D'Souza et al., 2009; Farshadfar et al., 2000; Jäger, 2010). The primary cause of rising mean temperatures is the enhanced level of atmospheric CO_2, which in itself could have a favourable effect on agricultural production. A higher concentration of CO_2 could moderate the yield-reducing effect of abiotic stress factors such as high temperature and water deficiency (Bencze et al., 2007; Varga and Bencze, 2009; Veisz et al., 2005).

The aim of the present work was to look for correlations between the duration of drought and the phenological, physiological and yield parameters of winter wheat. It was planned to determine how enhanced CO_2 levels affected the stress sensitivity of plants and whether they contributed to counteracting the damaging effects of drought.

Materials and methods

The experiments were carried out in the phytotron of the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár. Studies were made on two winter wheat (*Triticum aestivum* L.) genotypes with diverse genetic backgrounds, Mv Regiment (REG) and Mv Mambó (MAM). Mv Regiment is a high-yielding, soft-grained, intensive wheat variety, requiring optimum conditions to achieve its yield potential of 8–9 t/ha. It has good resistance to abiotic stress, surviving cold winters and heading early, thus allowing it to ripen before the most intensive heat and drought occur. Mv Mambó is an early-ripening, good quality hard-grained bread wheat, giving exceptionally high yields. It has excellent adaptation to weather conditions and a high level of frost resistance.

The plants were grown in two PGV-36 growth chambers (Conviron Ltd., Canada), both using the 't2' (Spring2) and 'ny2' (Summer2) climate programmes developed for winter wheat (Tischner et al., 1997), but one set to the normal ambient CO_2 concentration of 380 μmol mol^{-1} (NC) and the other to an enhanced level of 750 μmol mol^{-1} (EC). After 42 days of vernalisation at 4°C , winter wheat seedlings were planted four to a pot in 3000 cm^3 of a 3:1:1 (v/v) mixture of soil, sand and Vegasca (a humus-containing additive manufactured by Florasca, Hungary). The pots were watered daily and nutrient solution was added twice a week until the start of the treatments. The effect of drought was investigated in three phenophases, first node appearance (FNA), heading (H) and grain filling (GF), by withholding water completely for 7 or 14 days, resulting in a drop in the volumetric water content of the soil from 20–25% in the control to 3–5%, resulting in severe water deficiency symptoms in the plants.

The effect of drought on plant development was also investigated by comparing the heading dates of treated and control plants. The effect of water deficiency in various phenophases was studied by measuring changes in the grain number, grain mass and thousand-kernel mass. The intensity of assimilation processes has a decisive effect on the yield, so photosynthetic parameters were also recorded using a Licor-6400 gas analyser (portable photosynthesis system manufactured by the Licor Ltd., USA) on treated and non-stressed plants.

The combined effect of drought, the duration of the dry period and enhanced atmospheric CO_2 level on the grain protein content was determined using the Kjeldahl procedure (ICC Standard Method No. 105/2). The conversion factor of nitrogen to protein was 5.7 for all the samples. All the chemicals used were of analytical reagent grade.

Two-factor analysis of variance was applied to reveal significant differences between the data.

Results

Yield parameters

Of the two genotypes tested, the heading date of Mv Mambó plants subjected to water deficiency at first node appearance was modified by enhanced atmospheric CO_2. Heading was accelerated by the higher CO_2 concentration and by the longer period of drought. The plants headed 71 days after planting, four days earlier than at normal CO_2 concentration, where the negative effect of drought stress was not counterbalanced by the positive effect of surplus CO_2.

In non-stressed plants of Mv Mambó the grain number per plant was 34.3% higher at an atmospheric CO_2 concentration of 750 μmol mol^{-1} than at the normal level. At normal CO_2 level there was a greater decline in the grain number when the longer period of drought was applied at first node appearance (20.2%), while at enhanced CO_2 the grain number declined to the same extent (16%), regardless of the duration of drought in this phenophase (Fig. 1). When water was withheld at heading, the grain number decreased at both concentrations, but to a lesser extent at the enhanced level (25% after the longer period of drought, compared with 37.5% at normal CO_2). In the case of Mv Regiment, the grain number declined to the greatest extent when water deficiency was simulated at first node appearance, by 9.3 and 34% at normal CO_2 after 7 and 14 days of drought, respectively, and by 24.6 and 36.8% at enhanced CO_2 (Fig. 1).

When analysing the correlation between grain mass, the duration of drought and the atmospheric CO_2 level, it was found for both varieties that drought had a greater influence on the grain mass than CO_2 when the stress was applied at first node appearance, while the effect of the CO_2 level was greater in the case of water withholding at heading. After two weeks of drought simulation, the grain mass of Mv Regiment decreased by 71.3% at normal CO_2 and by 64.9% at the enhanced level (Fig. 2).

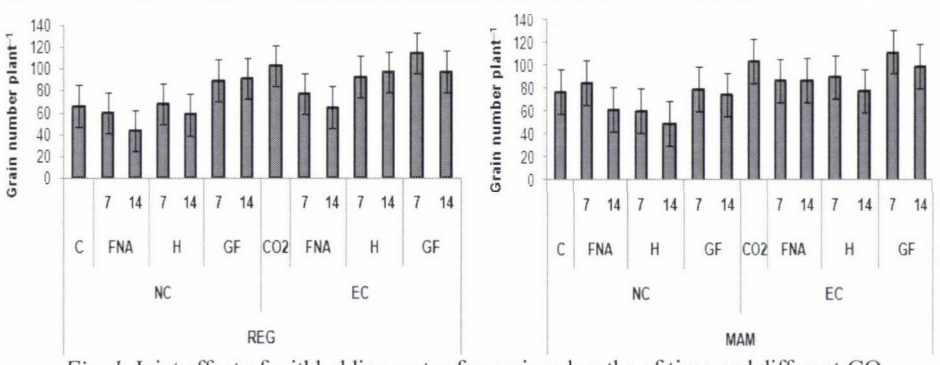

Fig. 1. Joint effect of withholding water for various lengths of time and different CO_2 concentrations on the grain number of Mv Regiment (REG) and Mv Mambó (MAM). Treatments: C: control, 380 μmol mol^{-1} CO_2 (NC); CO_2: control, 750 μmol mol^{-1} CO_2 (EC); 7: 7 days of drought stress; 14: 14 days of drought stress at 380 μmol mol^{-1} CO_2; FNA: first node appearance, H: heading, GF: grain filling ($LSD_{5\%}$= REG: 18.87; MAM: 19.22)

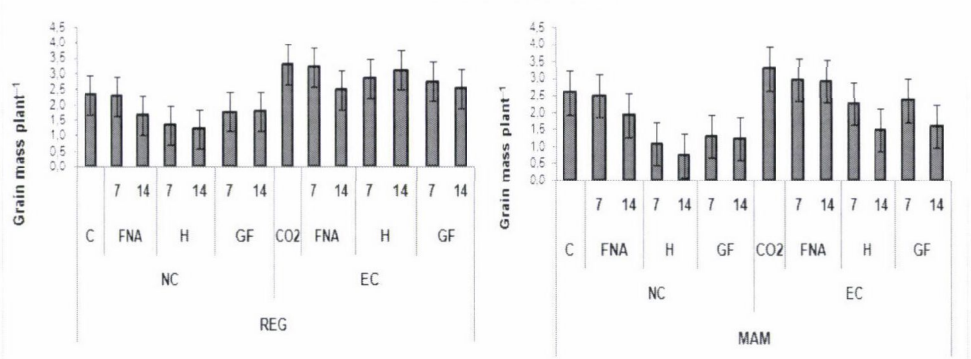

Fig. 2. Joint effect of withholding water for various lengths of time and different CO_2 concentrations on the grain mass of Mv Regiment (REG) and Mv Mambó (MAM) ($LSD_{5\%}$= REG: 0.63; MAM: 5.32). For treatments, see Figure 1

In Mv Mambó the negative effects of withholding water for 7 days at ripening were compensated by an increase in the atmospheric CO_2 concentration, but when the stress was of longer duration there was no detectable difference between the two CO_2 levels. Mv Regiment, on the other hand, was able to exploit the advantages of enhanced CO_2 even during a longer period of drought.

The effect of drought on the thousand-kernel mass only differed for the two varieties when the treatment was applied at first node appearance. In the case of Mv Mambó the thousand-kernel mass was reduced by 10% compared with the control at normal CO_2, whereas an increase of 13–15% was observed at enhanced CO_2, probably because the reduction in grain number was associated with an increase in grain mass (Fig. 3). Mv Regiment exhibited an increase in thousand-kernel mass in response to water withholding at first node appearance in all the treatments. When drought was simulated in later stages of plant development, it consistently resulted in a decrease in thousand-kernel mass, though differences could be detected both for the duration of stress and for the CO_2 concentration. The longer period of drought at heading did not cause any substantial reduction in the thousand-kernel mass compared to the 7-day treatment. Drought at maturity resulted in a similar decline to that observed for drought at heading. The effect of enhanced CO_2 was less pronounced, but was still detectable (Fig. 3).

Protein content

When drought was applied at first node appearance, only the longer period of stress led to a rise in the protein content. When applied at heading, both drought treatments increased the grain protein content by over 30% in Mv Regiment grown at normal atmospheric CO_2 level (Fig. 4). A similar rise in protein content was observed at normal CO_2 when water was withheld during the ripening period, and stress during this phenophase also led to an approx. 10% increase in protein at enhanced CO_2. Mv Mambó responded similarly to drought

stress at first node appearance, but drought at heading caused a substantial increase in the protein content even at the higher CO_2 level (Fig. 4). When drought was suffered during ripening at normal CO_2 the protein content was similar to that observed for Mv Regiment, but at enhanced CO_2 there was an increase of 20–30% in the protein content after 14 days of drought stress compared with the shorter treatment. In both varieties, however, the protein contents recorded at enhanced CO_2 were lower than for the corresponding treatment at normal CO_2. This difference was most pronounced when water was withheld at heading, with values 25–28.5% lower in Mv Regiment and 23–26% lower in Mv Mambó when the CO_2 concentration was increased. When drought was applied during ripening, this discrepancy was 20–22%. An increase in the duration of drought stress had the greatest influence on the protein content when applied at first node appearance.

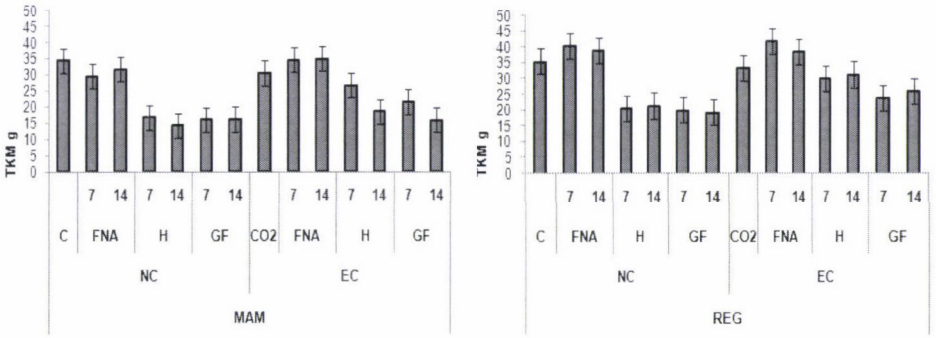

Fig. 3. Joint effect of withholding water for various lengths of time and different CO_2 concentrations on the thousand-kernel mass (TKM) of Mv Regiment (REG) and Mv Mambó (MAM) ($LSD_{5\%}$= REG: 4.08; MAM: 3.80). For treatments, see Figure 1

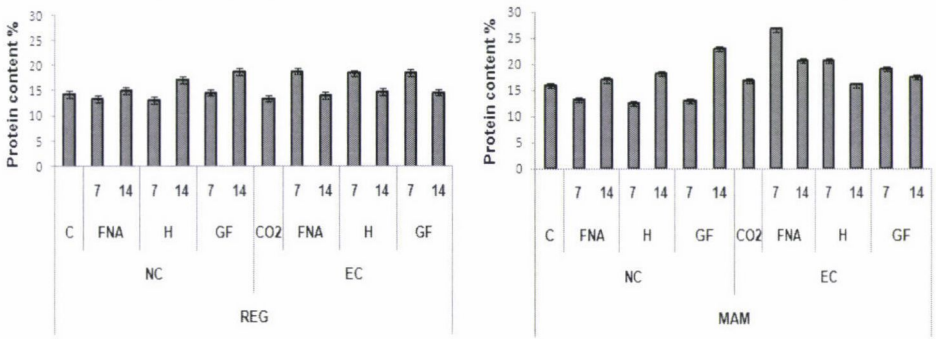

Fig. 4. Joint effect of withholding water for various lengths of time and different CO_2 concentrations on the protein content of Mv Regiment (REG) and Mv Mambó (MAM) ($LSD_{5\%}$= REG: 0.24; MAM: 0.35). For treatments, see Figure 1

Photosynthetic intensity

In the case of normal water supplies, the photosynthetic intensity of Mv Regiment was similar at the two CO_2 concentrations in all three developmental phases. When the soil moisture content at first node appearance dropped below 10%, however, there was a significant decline in the intensity at normal CO_2, while at the higher CO_2 concentration there was no detectable decrease in assimilation even at a soil moisture content of 5.1–10% (Fig. 5). The effect of drought at heading was again more pronounced at the normal CO_2 level, with a significant drop in photosynthesis in response to even a slight reduction in soil moisture. In the case of more severe drought, however, the decrease in gas exchange intensity was similar at both CO_2 levels. When water was withheld in the ripening stage, there was little difference between plants grown at the two CO_2 levels, a significant decrease in photosynthesis only being detected when the soil moisture content dropped to below 5%.

In Mv Mambó the intensity of photosynthesis was greater in all three phenophases when the CO_2 concentration was increased, with values considerably higher than those recorded for Mv Regiment. When drought occurred during ripening, the assimilation of Mv Mambó declined later and to a lesser extent than that of Mv Regiment, indicating that this genotype made better use of surplus CO_2 after heading.

The photosynthesis of Mv Regiment was negatively affected even by a slight reduction in the soil moisture content, while this was only observed after more severe stress in the case of Mv Mambó. The values of photosynthetic parameters decreased as the soil dried out during treatment. At 6% soil moisture there was a 70–90% reduction in photosynthetic activity compared to plants grown with normal water supplies at the same CO_2 level. The assimilation decreased to a greater extent at enhanced CO_2, which could be attributed to the fact that the level of photosynthesis was originally higher than at normal CO_2, but both dropped to around the same level when the soil dried out. When drought was experienced during ripening, the photosynthetic activity declined to a lesser extent than in the earlier phases of development. Higher values were recorded even when the soil became very dry (Fig. 5).

Discussion

Many authors have reported that an elevated level of atmospheric carbon dioxide results in an increase in plant height and yield (Kimball, 1983; Kimball et al., 1995; Cure and Acock, 1986). This can be explained by a reduction in stomatal resistance and an improvement in water utilisation (Morison, 1985). Modelling by Ewert et al. (2002) demonstrated that the yield-increasing effect of higher CO_2 levels was more intensive if it was associated with water deficiency. When subjecting plants to drought in various phenophases, Dickin and Wright (2008) found that water deficiency during ripening had the greatest effect on the yield of winter wheat. In the present work, drought was found to cause a

reduction in the grain number per plant, which was most sensitive to drought at first node appearance, while the correlation between grain mass reduction and water deficiency was closest when the latter occurred during ripening. The longer period of water withholding at ripening caused a 27.1–27.3% decrease in the grain number at normal CO_2, but the higher CO_2 level was able to compensate for this.

Leilah and Al-Khateeb (2005) found that the spike number, grain mass, harvest index and biomass quantity were the most important parameters for the analysis of drought effects, as these exhibited the closest correlation with changes in the available water reserves. In the present experiments, the longer period of drought at first node appearance had an outstanding effect on the grain mass. Compared with 7 days of water withholding, the more intensive water deficiency resulted in a further 0–23.4% drop in the grain number. When drought was simulated in the heading and ripening stages, a significant reduction in grain mass in response to an increase in the duration of drought was only observed at the enhanced CO_2 level. At normal CO_2 the values dropped to very low levels even after 7 days of drought.

Grain mass and thousand-kernel mass are recommended as criteria for selecting breeding materials capable of achieving high yields even in a dry climate (Li et al., 2001). These authors found a close correlation between drought stress and grain mass, with an increase of 2.1–2.3 mg in the grain mass in drought-stressed plants raised at enhanced CO_2 compared with those grown at the ambient level, while carbon dioxide had no effect on the grain mass in the case of normal water supplies. In the present work, water withholding at first node appearance resulted in higher grain mass per plant, but there was a decline in the thousand-kernel mass. In response to abiotic and biotic stress, Rosyara et al. (2009) reported reductions in both the yield and the thousand-kernel mass, but observed no changes in other yield parameters. By contrast, in the present work water withholding in the early stage of development resulted in a decrease in the grain number.

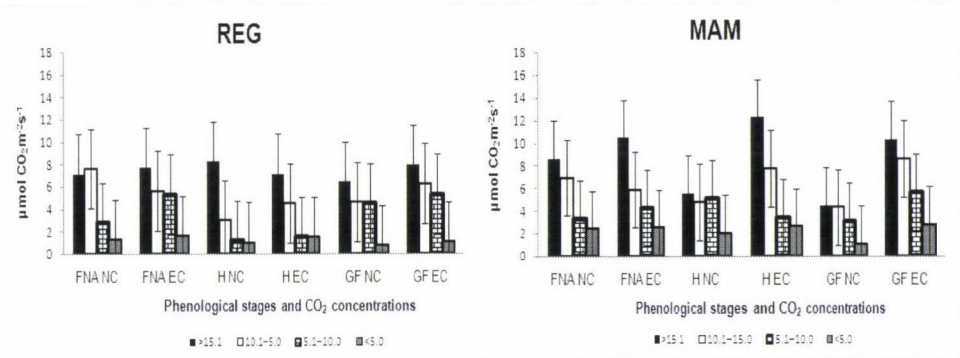

Fig. 5. Joint effect of soil moisture content and different CO_2 concentrations on the photosynthetic intensity (µmol CO_2 m^{-2} s^{-1}) of Mv Regiment (REG) and Mv Mambó (MAM) ($LSD_{5\%}$= REG: 3.21; MAM: 3.96). For phenological stages and CO_2 concentrations, see Figure 1

An increase in the protein content of spring wheat was demonstrated in response to drought by Mkhabela et al. (2010). Protein content was found to be in close positive correlation with the potential evapotranspiration and in negative correlation with the rainfall quantity. Consequently, the protein content was inversely proportional to the yield. A close correlation was found between yield quality parameters and drought during the period from planting to flowering. In the present investigations, the quantity of protein was modified primarily by drought during the period up to heading; when water was withheld at ripening the duration of drought had no influence on the protein content. Without drought stress, lower protein contents were detected at the higher CO_2 level, but the protein contents recorded at enhanced CO_2 after 14 days of water withholding were similar to those obtained at normal CO_2 with normal water supplies.

It was reported by Wall (2001) that enhanced CO_2 level reduced the damage to wheat under dry conditions, which could be attributed to the fact that a 200 μmol mol^{-1} increase in the CO_2 concentration resulted in a 30% increase in carbon supplies to the wheat leaves (Wall, 2001; Wall et al., 2000). Among the plant parameters, the root mass, leaf area and leaf thickness were influenced to the greatest extent by the CO_2 level. In the present work, an increase in assimilation was detected at the enhanced CO_2 level in non-stressed plants. In response to water withholding at first node appearance carbon fixation declined at a slower rate at elevated CO_2 than at the normal concentration and only after the soil moisture content had dropped to a lower level. Plant assimilation also remained higher at elevated CO_2 when water was withheld during heading and ripening.

The varieties tested have good tolerance of drought, with only slight modifications in the yield parameters in response to stress. The yield and physiological parameters of Mv Regiment, however, deteriorated substantially as a result of water deficiency, though this variety was better able to utilise surplus CO_2, giving outstanding results at elevated CO_2 level.

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INTEGRATION OF MOLECULAR GENOMIC DATA INTO THE MARTONVÁSÁR BREEDING INFORMATION SYSTEM

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In recent years an information system has been elaborated and constantly improved in Martonvásár, making it possible to handle the 3–4 million identification, observation, measurement, pedigree and other data generated for a total of almost 100,000 experimental plots each year. The extremely rapid development of biotechnology has made breeders interested in integrating molecular breeding methods into the conventional phenotype–pedigree system. The aim is to improve the competitiveness of breeding programmes through the intensive use of this new technology, with particular emphasis on determining how marker-assisted selection can be utilised. The present paper outlines not only a new data structure introduced to accommodate the new data elements of data categories such as gene sources, primer bank, primer combinations, markers, genes and alleles, but also data management tools and a standalone software interface to combine both molecular and phenotypic data. The integration of the molecular genomic data (GENETECH) with the information from the existing databases: pedigree (PEDIGREE), gene bank (GENEBANK) and germplasm exchange (GERMPEXCHG), ensures that biotechnological data generated at no little cost can be harnessed in ways that are important for breeders in decision-making. This is achieved through: (i) identification and centralization in uniform sources of the molecular data, and their matching with specific phenotypes, with special regard to those of importance for marker-assisted selection, (ii) integration and compliance with existing information system data, (iii) facilitation of decision- making based on the above (e.g. grouping of selection/crossing partners).

Key words: molecular breeding, marker-assisted selection, MAS, genomic database, breeding software

Introduction

The breeding cycle applied for the 76 wheat varieties so far developed in the Martonvásár wheat breeding programmes (Bedő, 2009) begins with the crossing of two parents and ends with the generation in which the selected advanced lines are judged by the breeder to be sufficiently homogeneous and

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competitive to be granted state registration as varieties or to be used as parents in new crosses. The breeding and field experimentation strategy, as interpreted in the present paper, includes all the data and information generated over a complete cycle.

The first steps in the computerisation of wheat breeding in Martonvásár were taken in the mid-eighties, when the first program modules were developed for basic breeding functions (crossing, selection, experimental designs) (Láng et al., 2001). Later, the number of such program modules gradually increased, with more and more tasks being linked to IT devices, leading to the natural desire to use the data to extract information that would improve the quality of decisions. The elaboration of the Martonvásár wheat breeding information system was the result of this strategy.

The aim of any information system is to connect users with sources from which they can extract the information they require (Burt and Kinnucan, 1990). The system described here can be used to store databases covering the whole breeding material, the institute's cereal gene bank, materials intended for germplasm exchange and the relevant pedigrees (Kuti et al., 2006), to plan field and laboratory experiments, to collect experimental data online, and to rapidly evaluate the experiments.

The new technology and the steep increase in the number of molecular marker-assisted analyses led to the appearance of new lines of investigation and many novel types of data. In addition to the molecular data available over the Internet ([1]), an increasing number of data are being generated within the institute by exploiting molecular genetic techniques.

One of the research programmes underway in the institute aims to improve stress resistance by applying molecular marker-assisted selection and the pyramiding of resistance genes to incorporate designated resistance genes into varieties adapted to Hungarian conditions and to select resistant phenotypes. The development of wheat varieties that are resistant or only slightly susceptible continues to be top priority (Vida et al., 2008). There is also a long tradition of testing the frost resistance of advanced lines in the phytotron before entering them in state variety trials (Veisz et al., 2001). The application of molecular genetic methods has also led to the compilation of linkage maps for two barley varieties (Karsai et al., 2007). Analysis using molecular markers generates new types of data (Uhrin et al., 2006), which could facilitate the more effective achievement of breeding aims, provided they can be successfully integrated with conventional breeding methods (Bedő et al., 2007).

The data model compiled around 10 years ago was not suitable for the handling and storage of molecular data, so the linking of these data with phenotypic data and their use in breeding was inadequate.

The elaboration of the molecular data model and the gene technology database ensures the storage and flow of information related to molecular marker-based selection, providing a reliable source of information for analysing

the genetic background of lines involved in crossing programmes. Instead of navigating through available data sources and manually combining the information obtained, with the help of the integrated system it will be possible to group/select gene sources in such a way that a higher proportion of lines satisfying the given breeding aims will be generated.

The basic functions of an information system can only be executed to an adequate standard if satisfactory hardware and software are available (Herdon, 2009). The handling of a large volume of data can be substantially simplified if the data are stored in separate databases, which also facilitate the intelligent processing and evaluation of the results. The most frequently employed relation models involve two-dimensional tables that take the logical structure of the stored data into account (Hernandez, 2003).

Materials and methods

Hardware requirements

The hardware requirements of the wheat breeding information system used in Martonvásár are ideally served by a range of computers with different capacity. The computers can be divided into two fundamental categories: workstations and data collectors.

Around 35–40 computers with greater capacity (Intel Dual Core E6300, 10/100 LAN, 2 GB DDR2 RAM, 250GB S-ATA HDD) are used as workstations for the programmers and users (researchers and scientific assistants) of the information system.

A further 20–25 computers, which are still functional but whose capacity is too small for use as workstations, are employed for automatic data collection. All that is required from these computers is that they should support the current Windows operational system (XP) and should have a connection to the local area network (LAN).

The operation of the information system also requires network tools to facilitate access to jointly used resources (databases, files, printers, etc.). The services and data available within the information system can be accessed by users through the institute LAN. The network data server provides simultaneous access to all the computers connected to the system (50–60 in peak hours).

Software requirements

Cooperation between the various software components makes the information system easy to use. Of the several hundred programs and system programs that perform various functions within the system, those essential for the operation of the system will be outlined below.

The application *Breeder*, which coordinates and operates the information system, is a Microsoft Windows-based software product, which can be run on any Windows-based operational system (Windows ME®, Windows® 2000, Windows XP®) and requires around 10 MB free disc space. It was developed using the Microsoft® Visual Basic Integrated Development Environment (VB-IDE) (Aitken, 1999), which occupies approximately 86 MB on the computer used by the program developer, but is not required by the users.

The majority of the output and input data used by the *Breeder* application are in a form that can be displayed using the Microsoft® Excel, Microsoft® Access and Microsoft® Word applications included in the Microsoft Office2000® and OfficeXP® program packages that can be run in the Windows® system (Jamsa and Klander, 1998). The Office program package (approx. 150 MB) must thus be installed on the work stations.

When choosing the operation system, software development tools and software that cooperate with the *Breeder* application, the available IT background (hardware/software) was taken into consideration.

Results

Data model

As in the case of all new research topics, the appearance of molecular genomic data raised the need first for their storage and handling, and later for their intelligent processing and efficient evaluation. To start with, when the quantity and applicability of this new type of data was far smaller than it is today, the data were stored as simply and rapidly as possible in a single data table, using the Word, Excel or, where possible, database-handling programs. In many cases all the data were included in a single file, which thus consisted of innumerable rows and columns, making them almost impossible to interpret.

As the application of marker-assisted selection became increasingly widespread, the demand arose for a more differentiated and efficient way of handling and utilising the results of this new technology. It became necessary for different groups of data to be handled separately and for the natural relationships between them to be apparent at the data level, so that the genetic background of phenotypic traits could be directly and rapidly recognised. To this end, a molecular data model had to be designed, which could be integrated into the breeding information system, after which software modules had to be written to cope with data handling, intelligent data analysis and the maintenance of links between the two systems.

It is clear from the depiction of the molecular data model (Fig. 1) that the various groups of data (Gene, Allele, Marker, PrimerBank) are clearly distinguishable from each other. The tables used to construct primer pairs and combinations (PrimerPairs, PrimerComb) have links to the markers they identify. The many-to-many relationships that exist between markers and alleles can be traced using a cross-reference table (MarkerForAllele). The data model also contains a gene synonym table (Synonym) and an extremely important table (DNASource), which provides a connection to the breeding database (not depicted in Fig. 1). This links up with the specific phenotype, from which it is possible to access (from both directions) all the information available for the given gene source: complete breeding, pedigree, gene bank and germplasm exchange data, together with qualitative and observational data, if these exist.

Software module

The integration of the molecular data model into the breeding information system led to the achievement of three interrelated aims:

1. New molecular data collected from the literature (Internet) now have their own place within a centrally accessible, uniformly utilisable data structure.

2. The rapidly growing number of results produced by the Institute's various departments from molecular analyses can be recorded in such a way that they link up with the phenotypes in the breeding database and can be simply accessed at any time.

3. Later, specialists (breeders) can apply this knowledge in the breeding area to make more informed decisions.

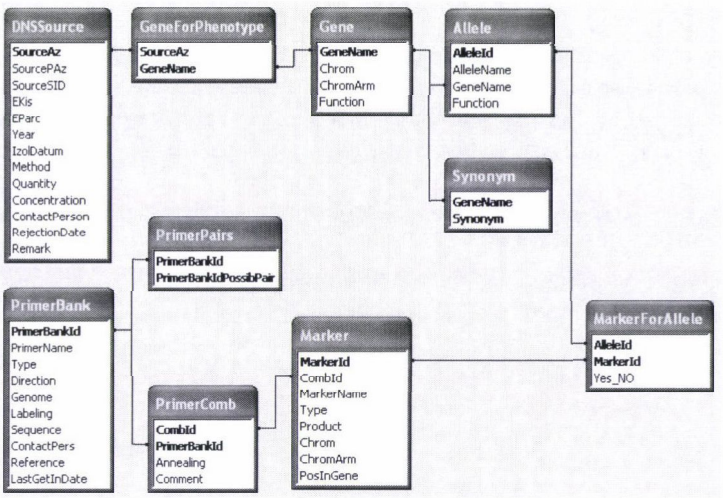

Fig. 1. Molecular data model

1. The software surface designed for the handling of molecular data (Fig. 2) allows the input/deletion/modification of data from various sources in the relevant categories (Gene, Allele, Marker, PrimerBank). In addition to the data handling functions available uniformly for all the categories, the PrimerBank category also allows the primers to be arranged in pairs. This means that reverse primers can be paired with each selected forward primer, or these pairings can be deleted.

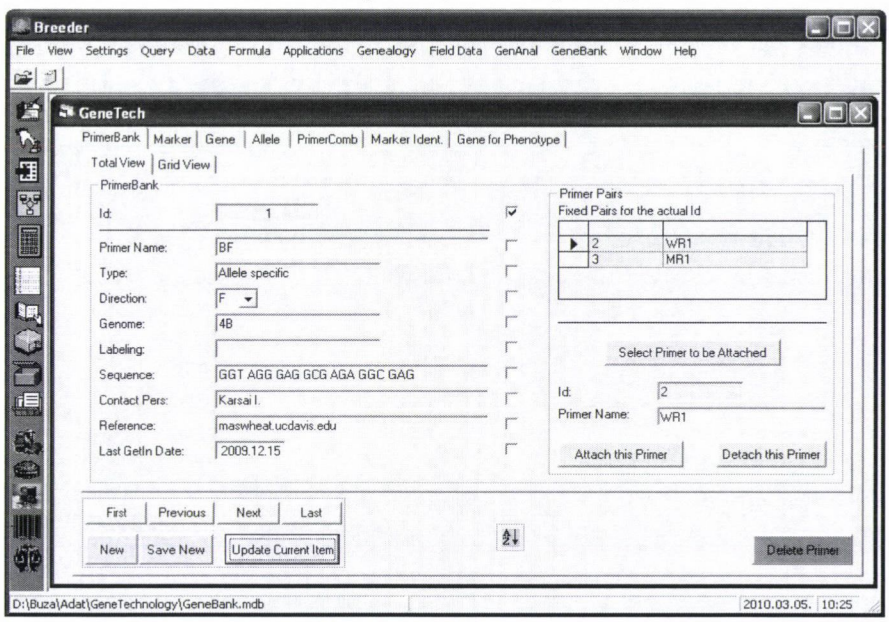

Fig. 2. Software module for molecular data processing

Various primer combinations are designed and tested to detect the genes in genotypes of interest. The system includes a separate function for the creation and display of these combinations (PrimerComb). As the PCR annealing temperature must be the same for both primers involved in the combination, this information can be entered in the Annealing field, while any special requirements for the reaction can be entered in the Comment field. When combinations are compiled, the system automatically informs the user of any markers previously identified using the given combination (Identified Markers list). Another important function is the linking of the primer combination to other closely related categories (Marker Ident. function). Depending on the number and type of data available, links may be made to one or more categories simultaneously. If a known marker can be identified using the chosen primer combination, this can be selected from the marker list automatically appearing on the screen, and the link can be saved by pressing the Save button. The same primer combination can be linked to any number of markers.

If the gene or allele linked with the primer combination is known, the names of these can be given in the Gene Name or Allele Name fields, and these will also be saved together with the primer combination. In the case of gene names, the system uses uniform nomenclature, but also makes use of the synonym list. This means that if the user enters a synonym of the name used by the system, this will be recognised, and the system will inform the user of the name under which the link has been stored.

2. Molecular breeding techniques allow the identification of genes important for breeding purposes, their localisation on the genome and selection for these genes. This can be achieved most reliably using own results originating from molecular analysis based on Internet and own data. The results of such analyses can be recorded using a function specially designed for this purpose (Gene for Phenotype function). The first step is to create a link between the primer combination selected for the testing of the desired gene source and the markers identified by the PCR analysis (Marker Ident. function).

If the tests identify markers suitable for the detection of a known gene or allele, this fact is recorded, indicating the name of the gene or allele (if known) associated with a specific marker. The results of PCR analysis reveal the size of product given by the tested primer combination on the individual genotypes, which can be identified as a known gene or allele (if the latter exists). The final step is to link up the gene sources with the markers identified. This can be done of the basis of the results of PCR analysis (product size) using the Gene for Phenotype function. When the name of the desired experiment (gene source) is selected, the whole of the tested material appears on the screen. If the primer combination used for testing is selected, the markers it identifies are automatically displayed. By clicking on the gene source and the relevant marker (on the basis of product size) a direct link between the tested genotype and the genes/alleles it carries can be created and saved (Fig. 3) in such a way that all the relevant information (primer pair/combination, marker, methodological description) is instantly accessible.

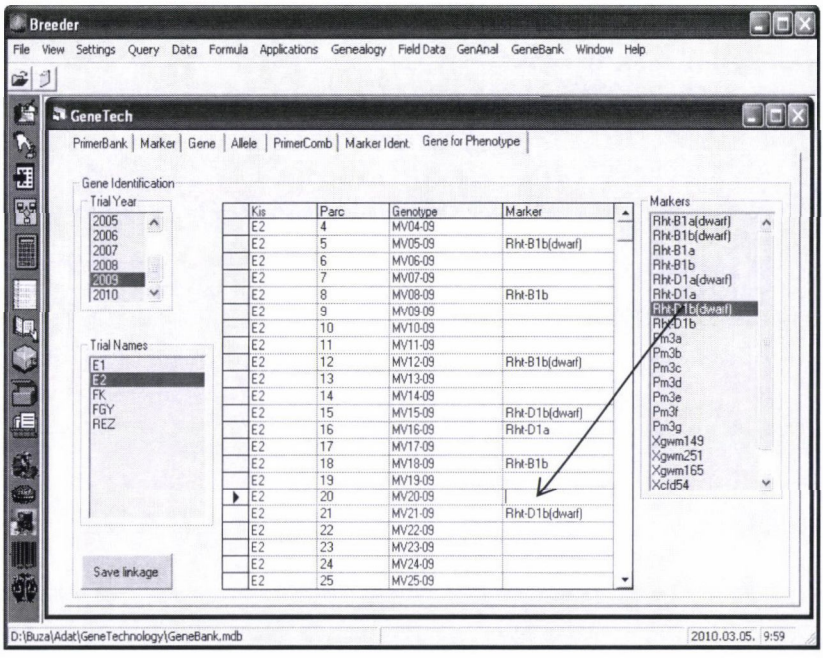

Fig. 3. Combining molecular and phenotypic data

3. The collection of molecular genomic data (from the literature, the Internet and own results) and their storage in a centralised system provides the necessary conditions for the achievement of the main aim of applied genomic research: the determination of the functions of identified genes and their utilisation in plant breeding. In order to assure full usability of the molecular genomic data to users of the breeding information system, a new module has been developed to integrate molecular genomic data into the existing breeding information system (Fig. 4).

The following practical benefits accrue from the integration of the molecular genomic subsystem:

Early selection

Targeted experiments can be designed to provide a constant supply of methodological and analytical data for the identification of genes coding for useful agronomic traits and their markers, linking this information with specific phenotypes involved in breeding programmes. Centralised data storage and processing have the enormous advantage that the results of genetic analyses performed by various groups (scientists, technicians, lab assistants) and departments can be displayed within a single system, focussing on the main points of interest. This makes it possible to establish a gene profile for any given genotype, using all the genetic information stored in the system. This has several

advantages: (i) major traits can be distinguished with the help of molecular markers (if these are available) in early stages of development, thus allowing early selection to be made, (ii) it is simple for scientists to compare their own results with data from external sources, (iii) further analyses can be designed more accurately.

Selection of crossing partners

The integration of the genomic subsystem facilitates the screening of the breeding material for gene sources carrying known genes for desirable traits. This allows the breeder to choose parental combinations with a better chance of producing progeny generations carrying these genes. On the basis of the gene profiles generated from the available data, the system itself is able to make recommendations on optimum parental combinations for specific traits (genes).

Genetic purity, variety rights

In addition to morphological traits (DUS), molecular genetic markers are also applicable for the determination of genetic distinctness. The gene profiles compiled using the molecular data stored in the system (together with the relevant markers and methodology) greatly facilitate the design of tests on genetic purity. If preliminary information is available, SSR markers are particularly suitable for distinguishing between genetic materials and for the determination of genetic purity.

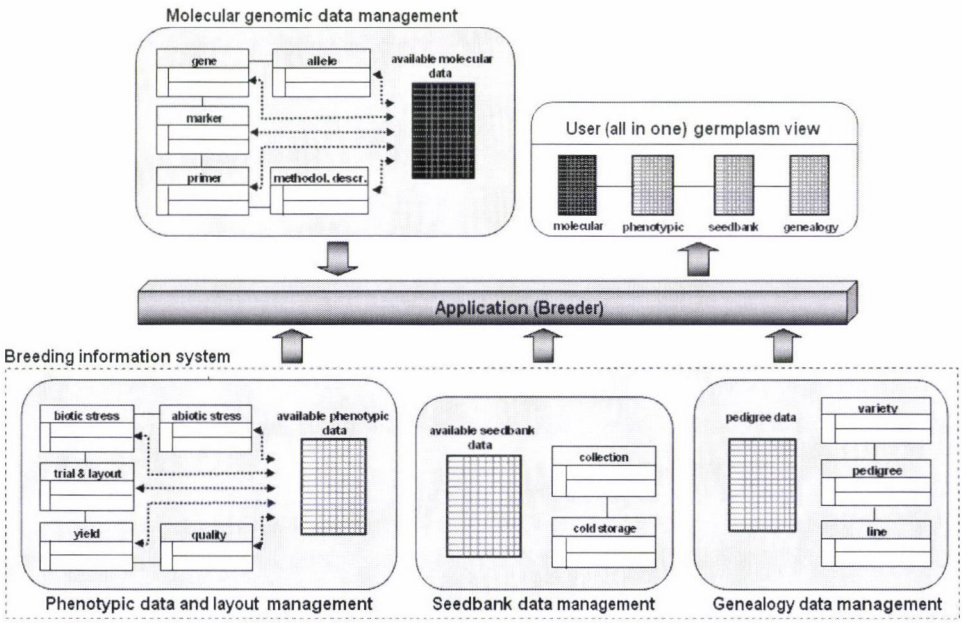

Fig. 4. Integration process overview

Discussion

The rapid development of molecular genetics and its application in practice (biotechnology) has made an important contribution to the improvement of agronomically useful plant traits and to the enhancement of stress resistance. This new technology is able to complement conventional breeding methods, the efficiency of which is also greatly enhanced by information technology. The compilation of data on molecular marker-assisted selection, from own research and from the literature, and their integration into earlier data models and pedigree registers, makes it possible to analyse the genetic background of major gene sources. With the aid of specific applications, this information reaches the breeder in the form of selection predictions, based on the ability of the IT equipment to rapidly identify the genetic background of desirable traits. Based on the specific information available in the gene technological database (parental partners), it is possible to predict what traits will be inherited, in some cases without phenotypic testing for the given trait. The access both to molecular and phenotypic data can improve the breeding process as it allows cereal breeders to find the right molecular markers to be used for the targeted breeding of certain traits.

The system described here could also be utilised in other research projects where the large volume of data on genotypes and markers makes it difficult to obtain a comprehensive picture using traditional methods.

The IT system that has been successfully used in Martonvásár for nearly three decades to support breeding activities by simplifying practical implementation and improving integration, information flow and cooperation, thus leading to better decisions and a higher standard of achievement, will be even more effective with the addition of the new molecular genomic subsystem, which is able to satisfy functional demands. IT thus continues to be an important auxiliary component in the breeding strategy.

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EFFECT OF HIGH TEMPERATURE AND DROUGHT ON THE COMPOSITION OF GLUTEN PROTEINS IN MARTONVÁSÁR WHEAT VARIETIES

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Finding and improving wheat cultivars with good adaptability to abiotic stress is an important objective in breeding programmes. An experiment was set up in the climate chamber of the Martonvásár phytotron to test the effect of heat and drought stress on two winter wheat varieties and one variety of durum. Wheat plants exposed to 35°C and drought during grain filling exhibited altered agronomic and grain quality characteristics. Drought was found to have a much greater influence on yield and quality than heat stress. Reductions in the unextractable polymeric protein fraction and the glutenin-to-gliadin ratio indicated poorer grain yield quality as a result of drought, despite higher protein content. Quality deterioration was observed after drought, while heat stress had no noticeable influence on the protein quality of the three wheat genotypes, measured using size exclusion high performance liquid chromatography (SE-HPLC). The durum variety had a better ratio of protein components and a significantly higher Zeleny value when exposed to heat stress, although it had the lowest grain yield and grain/straw ratio.

The most significant negative correlation was observed between the Zeleny value and the unextractable polymeric protein (UPP%) fraction after heat treatment and between the relative protein content and the albumin+globulin % (AG%) in the case of drought. These correlations testify that these parameters play an important role in determining the baking quality of wheat flour.

Key words: heat stress, drought, protein content, unextractable polymeric protein, glutenin-to-gliadin ratio, grain/straw ratio, grain yield

Introduction

Hungary has a very variable climate, with many extreme weather events, so it is essential to grow wheat varieties with excellent adaptability. In general varieties with good adaptability tend to have lower genetic yield potential than intensive varieties, but have better tolerance of unfavourable environments (Kondora et al., 2000).

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Wheat production in Hungary faces the challenge of extremely high temperature, combined with water deficiency, leading to substantial losses in both the quantity and the quality of the grain. In terms of cereal and food chemistry, better quality means a larger proportion of biologically active protein with a more favourable amino acid composition (Koltay and Balla, 1982). The quality of wheat, and of the flour obtained from it, is primarily associated with the gluten quantity and quality (Uri et al., 2006). The main constituents of gluten are gliadin and glutenin. The ratio of the two components is important, and a ratio of 25% glutenin to 75% gliadin is considered to be the most favourable. The gluten quantity is positively correlated with the protein content. Gluten quality and quantity are independently inherited, which means that a variety may have good quality even if it has a lower gluten quantity.

The development of gluten proteins can only be detected in wheat kernels 20–25 days after flowering, but a rapid increase in both the quantity of gluten proteins and the unextractable polymeric protein ratio was observed during the grain-filling period (Abonyi et al., 2010). The soluble fraction consisted mainly of gliadin and the unextractable fraction of glutenin. Other authors also reported that the formation of gluten took place in two steps: first the polymerisation of glutenin subunits, followed by the aggregation of glutenin polymers and gliadins. These processes were influenced by both genetic and environmental factors (Hamer and Van Vliet, 2000; Rhazi et al., 2003). Heat stress, as well as limited water availability, may significantly impair photosynthesis (Harding et al., 1990; Subrahmanyam et al., 2006), reducing the amount of assimilates available to the grain.

It was demonstrated by Zhao et al. (2009) that protein components are very sensitive to drought stress during the later stages of grain filling. The deterioration in dough quality could be attributed to reductions in the glutenin-to-gliadin ratio and the percentage of very large glutenin polymers in response to high temperature (Blumenthal et al., 1994; 1995; Bencze et al., 2004).

The aim of the present work was to determine how high temperature and drought at ripening were correlated with the yield quantity and quality of selected wheat varieties. The analysis included measurements on the protein content of stress-treated wheat plants and the ratio of protein components, the determination of changes in the grain/straw ratio of the total aboveground biomass, and a search for correlations between qualitative and quantitative parameters.

Materials and methods

The experiments were carried out in a climate chamber (Conviron PGV-36) in the Martonvásár phytotron, adjusted to simulate high temperature and drought stress, in order to investigate the effect of these factors on the quality components of the grain yield.

Two winter wheat (*Triticum aestivum* ssp. *aestivum*) varieties (Mv 15 and Mv Magma) and one durum wheat (*T. turgidum* ssp. *durum*) (Mv Makaróni) were compared in the experiment.

Mv 15, a medium late wheat variety which was widely grown for many years, has outstanding yield stability in its maturity group, primarily due to its heat tolerance. Mv Magma is a good quality, mid-early bread wheat, whose excellent yield potential can be attributed to its good resistance. Mv Makaróni, which is also mid-early ripening and has short red spikes, is a winter durum wheat with good yield potential.

Germinated wheat grains were planted, four to a pot, in 3.5 L pots containing a known quantity of a 3:2:1 mixture of soil, Vegasca and sand. After six weeks of vernalisation at 4°C, the plants were grown until the beginning of the stress treatment using a spring-summer climatic programme developed for winter wheat (Tischner et al., 1997).

There were 12 pots for each variety, four for each of the treatments: control (C), heat stress (H) and drought stress (D). Treatment was begun 12 days after heading (Zadoks 75) and was continued for 15 days. The temperature was 24/20°C (day/night) in the control chamber (Tischner et al., 1997) and 35/20°C in the heat-stressed chamber. The daytime temperature was programmed for 8 hours. The soil moisture content was adjusted in terms of natural water capacity, which was taken as 100% saturation. The control plants were kept at a value of 60–70% and the drought-stressed plants at 40–45%. Water was given on a weight basis. The light intensity during the stress treatments was adjusted to 350 μmol m^{-1} s^{-1}.

Two-factor analysis of variance was performed for the statistical evaluation of the data using the 'Breeder' program (Láng et al., 2001). Correlation analysis was carried out on the yield and quality data of the three varieties.

The grain protein content and the % distribution of the protein components were analysed. After harvest maturity was reached, measurements were made on the grain yield per plant, the thousand-kernel weight and the grain/straw ratio of the total aboveground biomass. The wholemeal required for the analysis of quality parameters was produced using a Perten 3100 Laboratory Mill.

Wholemeal samples from stress-treated plants were analysed in three replicates using a Perten Inframatic 8611 instrument, which works on the near infrared reflectance spectroscopy principle and uses very small sample quantities to determine the protein content and the Zeleny number (ICC, 1995). The latter provides a reliable prediction of the loaf volume. A low sedimentation value results in denser loaves with smaller volume.

Changes in the ratio of protein components in the wheat kernel were analysed in plants exposed to high temperature or drought. The measurements were carried out on 0.1 g samples in three replications. The total glutenin, gliadin and albumin+globulin contents of the samples were determined by separating the proteins on the basis of size, using the SE-HPLC technique, according to the method of Batey et al. (1991). A Phenomenex BIOSEP-SEC 4000 column was used for the separation and the proteins were detected at 210 nm. The total glutenin and gliadin quantities were expressed as a % of the control. The unextractable polymeric protein fraction (UPP%) was determined using the method reported by Gupta et al. (1993) as a ratio of the total polymeric protein fraction. Determinations were also made of the glutenin/gliadin ratio and the albumin+globulin %, calculated as the ratio of the soluble albumins and globulins to the total polymeric protein.

Results

The grain/straw ratio, an important criterion when choosing wheat varieties, may change considerably under stress conditions, so the total aboveground biomass was divided into total grain mass and total straw mass, and differences were recorded in response to heat and drought stress (Fig. 1). For all three varieties (Mv 15, Mv Magma, Mv Makaróni), heat and drought stress caused a decline not only in the total biomass, but also in the grain mass in comparison with both the straw mass and the grain/straw ratio of the control plants. Drought stress caused a greater reduction in the grain mass than heat stress in all three varieties.

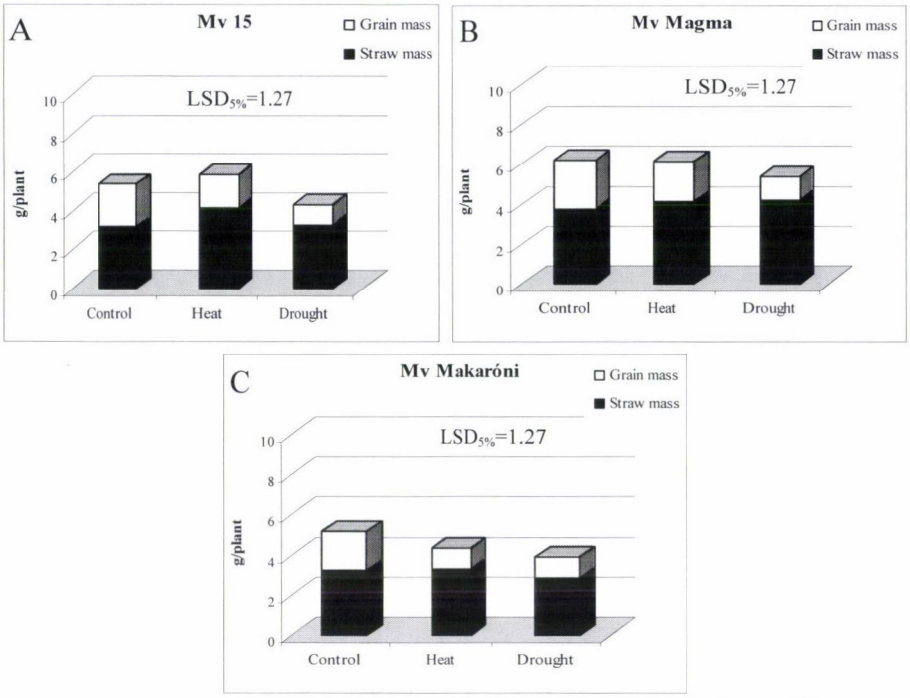

Fig. 1. Changes in the grain/straw ratio of the aboveground biomass in response to heat and drought stress in the varieties Mv 15 (A), Mv Magma (B) and Mv Makaróni (C) ($LSD_{5\%}$ in terms of total biomass)

As expected, the heat-tolerant variety Mv 15 responded well to 15 days of treatment at 35°C (Fig. 1A). There was no reduction in the biomass, and although there was a drop in the grain mass (22.7%), the straw mass increased compared with the control. Mv Magma also exhibited good tolerance of heat stress, producing the same quantity of biomass as the control plants (Fig. 1B). The straw mass increased slightly and the decrease in the grain mass was the smallest in Mv Magma (18.8%). Mv Makaróni, however, exhibited a substantial loss of grain mass in response to heat (46.5%; Fig. 1C).

Both heat stress and drought caused a reduction in the yield of all three varieties (Fig. 2A). In the case of Mv Magma and Mv 15, grain yield losses were lower after heat stress than after drought, with values of 18.8% and 52%, respectively, for Mv Magma and 22.75% and 53% for Mv 15 after heat and drought stress, respectively. Mv Makaróni exhibited similar yield losses after both heat (46.5%) and drought stress.

Heat stress also caused less pronounced changes than drought in the thousand-kernel weight of the varieties (Fig. 2B), with the greatest reduction (29.5%) in Mv 15. Mv Magma responded most sensitively to drought, with a 52% decrease in the thousand-kernel weight compared with the control, but there was also a 49% reduction in this parameter in Mv 15. Despite the great reduction in yield, Mv Makaróni proved to be most tolerant of drought in terms of thousand-kernel weight, with a loss of 32.5%.

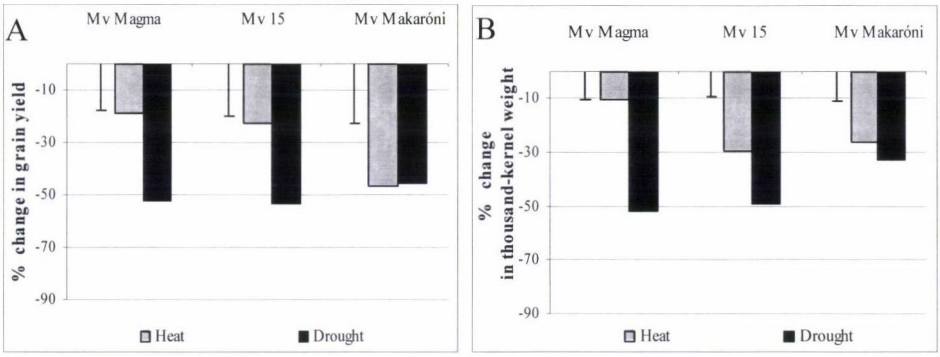

Fig. 2. Changes in the grain yield (A) and thousand-kernel weight (B) of the varieties in response to heat and drought stress, as a % of the control (0%) (Bar: differences significant at the P≤ 0.05 level)

In response to the stress treatments an increase in the relative protein content of the grain was observed in all three varieties (Fig. 3A), but again heat had less effect than drought. There was a rise of 15.3% in the protein content of Mv 15, but only 2.65% for Mv Magma after heat treatment, while drought caused the greatest change in Mv Magma (34.7%) and had a much milder effect on Mv 15 and Mv Makaróni (18 and 16%, respectively).

Both reductions and increases were detected in the Zeleny index (Fig. 3B). While both heat and drought stress led to reductions in Mv Magma, in the case of Mv 15 the Zeleny index increased after heat stress but declined after drought. For both varieties, however, only drought stress had a significant effect, while in the case of Mv Makaróni both treatments led to a significant increase in the Zeleny index (heat: 26.6%; drought: 39.6%).

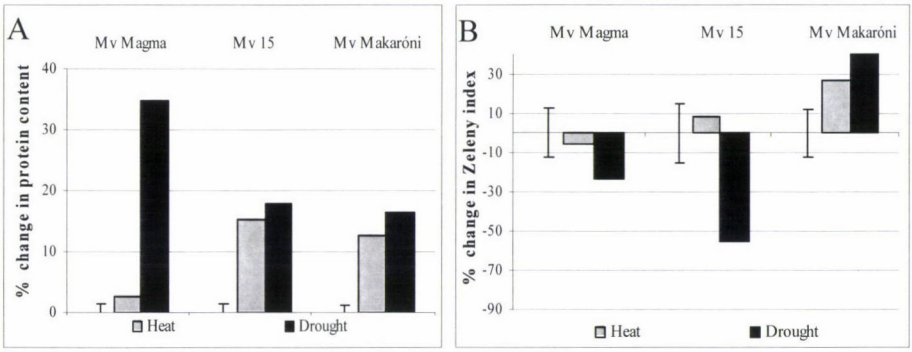

Fig. 3. Changes in the relative protein content (A) and Zeleny index (B) of the grain yield in response to heat and drought stress, as a % of the control (0%)
(Bar: differences significant at the P≤0.05 level)

It could be seen from the percentage distribution of the protein components that drought stress caused a significant increase in the total glutenin content of all three varieties compared to the control (Fig. 4A), with the greatest increase for Mv Magma, followed by Mv 15 and Mv Makaróni. Heat stress only increased the total glutenin quantity significantly in the case of Mv 15.

In two varieties, Mv Magma and Mv Makaróni, drought stress resulted in a significant rise in the total gliadin content compared with the control (Fig. 4B), while in the case of heat stress a significant increase in gliadin was observed for Mv 15 and Mv Makaróni.

Drought stress resulted in a significant reduction in the unextractable polymeric protein (UPP%) compared to the control (Fig. 5A) in all three varieties (Mv Makaróni: 19.45%; Mv 15: 15.86%; Mv Magma: 12.27%). Heat stress only led to a slight but significant increase in UPP% in the case of Mv Makaróni, having no detectable effect on the other two varieties.

A significant decrease in the glutenin-to-gliadin ratio compared to the control was only observed in Mv Magma after drought stress (Fig. 5B). Both heat and drought stress led to a significant increase in this ratio in Mv 15, while neither treatment resulted in any significant change in the case of Mv Makaróni.

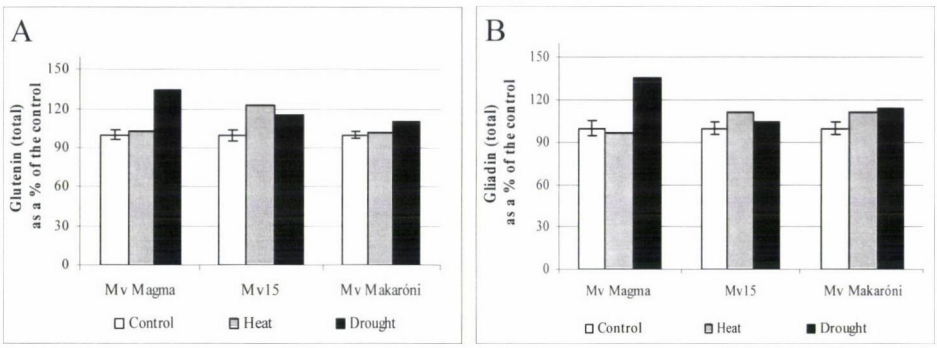

Fig. 4. Changes in the total glutenin (A) and total gliadin (B) contents in response to heat and drought stress, as a % of the control (Bar: differences significant at the P≤0.05 level)

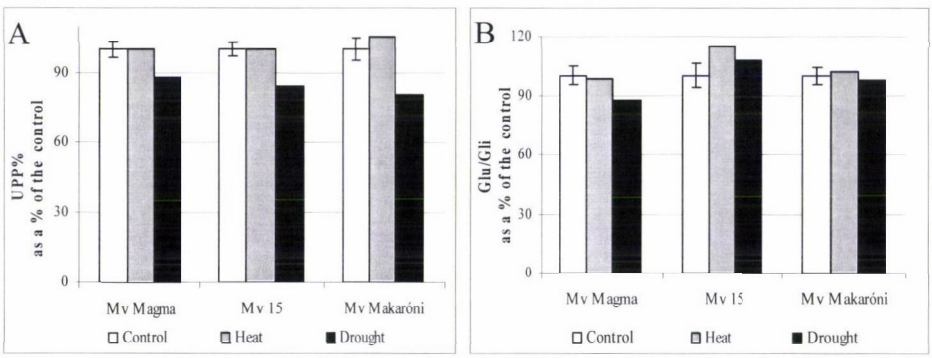

Fig. 5. Changes in (A) the unextractable polymeric protein (UPP%) and (B) the glutenin-to-gliadin ratio (Glu/Gli) in response to heat and drought stress, as a % of the control (Bar: differences significant at the P≤0.05 level)

In response to heat stress there was a significant rise in the albumin+globulin % compared to the control in both Mv 15 and Mv Makaróni (Fig. 6). In the case of drought stress, reductions were observed for Mv Magma and Mv Makaróni and a significant (12%) increase for Mv 15.

Correlation analysis revealed both positive and negative correlations between the quantitative and qualitative yield parameters of the three varieties (Table 1). In response to heat stress the closest significant correlations were detected between the Zeleny index and UPP% ($r = -1.00^{**}$), the grain yield and the harvest index ($r = 1.00^{**}$), and the total biomass and the grain number ($r = 0997^{*}$). Significant correlations were also observed between thousand-kernel weight and protein content ($r = -0.993^{+}$), grain mass and biomass ($r = 0.990^{+}$) and biomass and harvest index ($r = 0.994^{+}$).

Fewer significant correlations were detected between the quantitative and qualitative yield parameters of the three varieties in the case of drought stress (Table 2). The closest correlation ($r = -0.997^{*}$) was found between the relative protein content and the albumin+globulin %, followed by the biomass and the grain number ($r = 0.994^{+}$), the thousand-kernel weight and the harvest index ($r = 0.992^{+}$) and the UPP% and the Glu/Gli ratio ($r = -0.992^{+}$).

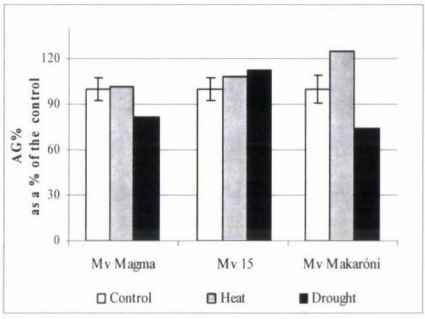

Fig. 6. Changes in the albumin+globulin % (AG%) in response to heat and drought stress, as a % of the control (Bar: differences significant at the $P \leq 0.05$ level)

Table 1

Correlation matrix for the yield and quality parameters of the three varieties (Mv Magma, Mv 15, Mv Makaróni) in the case of heat stress

Heat stress	GY	TKW	PC (%)	Z (ml)	UPP%	Glu/Gli	AG%	Biomass	Grain No.
TKW	0.97								
PC (%)	–0.94	-0.993^{+}							
Z (ml)	–0.91	–0.80	0.72						
UPP%	0.91	0.79	–0.71	–1.00**					
Glu/Gli	–0.72	–0.54	0.44	0.94	–0.94				
AG%	0.04	–0.19	0.30	–0.45	0.45	–0.72			
Biomass	0.990^{+}	0.93	–0.88	–0.96	0.96	–0.81	0.19		
Grain No.	0.97	0.90	–0.84	–0.98	0.98	–0.86	0.27	0.997*	
HI	1.00**	0.96	–0.93	–0.93	0.92	–0.75	0.08	0.994^{+}	0.98

GY: Grain yield; TKW: Thousand-kernel weight; PC: Protein content; Z: Zeleny; UPP%: Unextractable polymeric protein; Glu/Gli: Glutenin-to-gliadin ratio; AG%: Albumin+globulin %; HI: Harvest index; **, *, $^{+}$: Significant at the $P \leq 0.01$, 0.05 and 0.1 levels, respectively

Table 2
Correlation matrix for the yield and quality parameters of the three varieties (Mv Magma, Mv 15, Mv Makaróni) in the case of drought stress

Heat stress	GY	TKW	PC (%)	Z (ml)	UPP%	Glu/Gli	AG%	Biomass	Grain No
TKW	–0.87								
PC (%)	0.33	0.18							
Z (ml)	–0.06	0.54	0.92						
UPP%	0.42	–0.81	–0.72	–0.93					
Glu/Gli	–0.30	0.73	0.80	0.97	–0.992^{+}				
AG%	–0.25	–0.26	–0.997*	–0.95	0.77	–0.85			
Biomass	0.94	–0.98	–0.02	–0.41	0.71	–0.62	0.10		
Grain No.	0.97	–0.97	0.08	–0.31	0.63	–0.53	0.00	0.994^{+}	
HI	–0.80	0.992^{+}	0.30	0.64	–0.88	0.81	–0.37	–0.96	–0.93

For abbreviations, see Table 1. *, $^{+}$: Significant at the $P \leq 0.01$, 0.05 and 0.1 levels, respectively.

Discussion

High yields are only of real value if they are associated with good quality. When choosing which variety to grow, the tolerance of the genotypes to high temperature and drought should also be taken into consideration, together with the effect of these stress factors on the quantity and quality of the yield.

A comparison of the two types of stress revealed that heat stress (35°C for 15 days) had less effect on the parameters investigated than drought stress. A considerable change in the grain/straw ratio was observed after stress treatment. Both heat and drought stress caused a reduction not only in the total biomass, but also in the grain mass, compared with both the straw mass of the stressed plants and the grain mass of the control plants. In all three genotypes, however, the reduction caused by drought was greater than that resulting from heat stress.

Together with the decrease in the grain/straw ratio, there was also a decline in the grain yield and the thousand-kernel weight. Mv Magma had the best tolerance of high temperature, exhibiting the smallest changes in the grain/straw ratio, the grain yield and the thousand-kernel weight, while Mv Makaróni was the most sensitive, as both types of stress had a similarly drastic effect on the yield, the thousand-kernel weight and the grain/straw ratio.

The results published by Bhutta (2007) also revealed that drought stress had a drastic effect on the development and yield of plants. This author suggested that the flag-leaf area, germination in mannitol and the number of tillers per plant, which were in significant positive correlation with the grain yield, could be used as selection criteria for the identification of drought-tolerant wheat genotypes.

The present work indicated that, in addition to the above parameters, there were also significant correlations between the yield and the biomass ($r = 0.990^{+}$) and the yield and the harvest index ($r = 0.999$*) in the case of drought stress,

while significant correlations were detected between the biomass and the grain number after both heat stress ($r = 0.997^{*}$) and drought stress ($r = 0.994^{+}$).

Trends in quality traits can also be explained in the light of changes in the yield parameters. The rise in the protein content of the kernels could be predicted from the decrease in the thousand-kernel weight. Heat stress had the least effect on the thousand-kernel weight of Mv Magma, so the increase in the protein content was also less pronounced in this variety than for the other two genotypes. In the case of drought stress it was also observed that the greatest reduction in the thousand-kernel weight was associated with the greatest increase in the protein content (Mv Magma). The correlation matrix also confirmed the correlation between the thousand-kernel weight and the relative protein content in the case of heat stress ($r = -0.993$). Other authors also reported a significant rise in the grain protein content in response to heat stress (Wrigley et al., 1994; Corbellini et al., 1997; Daniel and Triboï, 2000; Bencze et al., 2004; Castro et al., 2007; Labuschagne et al., 2009; Hrušková and Švec, 2009).

An increase in the grain protein content is not necessarily associated with an improvement in protein quality, as this is also influenced by the ratio of protein constituents. When the total glutenin and total gliadin quantities were determined in kernels with increased protein content, it was found that the total glutenin quantity rose to a greater extent after drought than after heat stress. The only exception was Mv 15, where greater increases in total glutenin and total gliadin were recorded after heat stress. Gupta and MacRitchie (1994) also reported that the quality of bread is affected not only by the quantity of gluten, but also by the ratio of the protein fractions. Kasearu et al. (1997) suggested that the low gluten protein content of triticale flour could be responsible for the poorer quality of dough made from triticale.

Whether a rise in the relative protein content will have a favourable or unfavourable effect on the breadmaking quality depends not only on the protein components, but also on the Zeleny index. In general, a lower value of this index is indicative of a deterioration in grain quality despite the increase in protein content. The Zeleny index was only influenced positively by high temperature and drought treatment in the case of Mv Makaróni. In the other two varieties, the significant decline in the Zeleny index after drought indicated poorer quality.

The stress treatments also affect the unextractable polymeric protein percentage (UPP%) and the glutenin-to-gliadin ratio (Glu/Gli). The reduction in these parameters after drought stress caused deterioration in grain quality despite the rise in the protein content. With the exception of Mv Makaróni this was confirmed by a drop in the Zeleny index. However, when the plants were exposed to a temperature of 35°C during grain filling, there was no substantial decline in quality, as confirmed by the rising values of the Zeleny index. The correlation matrix for the three varieties also revealed a close negative correlation ($r = -1.00^{**}$) between the Zeleny index and the UPP% in the case of heat stress. Close negative correlations were detected between the UPP% and the

Glu/Gli ratio ($r = -0.992^{+}$) and the relative protein content and the albumin+globulin % ($r = -0.997^{*}$). The greater accumulation of protein due to the reduced thousand-kernel weight resulted in an increase in the albumin+globulin % in the case of heat stress. In Mv Makaróni this led to a significant increase in the UPP% and to more favourable values of the Zeleny index.

Zhao et al. (2009) also found evidence that the protein composition is sensitive to drought during grain filling, resulting in a deterioration in dough quality due to a reduction in the glutenin-to-gliadin ratio and the percentage of very large glutenin polymers.

Stone and Nicolas (1995) stated that the alteration in protein synthesis caused by the timing and duration of heat stress could be detected as a change in the glutenin-to-gliadin ratio. In the present study, the shift in this ratio due to heat stress was relatively small or statistically non-significant. This is in agreement with the findings of Castro et al. (2007), who reported that heat stress had no noticeable effect on the protein quality of six wheat genotypes, as measured using high performance liquid chromatography. It could be concluded that varieties where the grain characteristics and the ratio of protein components were relatively stable could be used as genetic sources for improving resistance to heat stress.

Acknowledgements

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EFFECT OF FARMYARD MANURE AND MINERAL FERTILISER ON THE GROWTH OF MAIZE (*Zea mays* L.) IN A LONG-TERM EXPERIMENT II. USING THE HUNT–PARSONS PROGRAM FOR PLANT GROWTH ANALYSIS

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In a long-term continuous maize experiment set up in 1959, the functional method of growth analysis was applied to investigate the effect of various levels of farmyard manure and mineral fertilisation on the growth of maize (*Zea mays* L.) and on the dynamics of the growth parameters over a 3-year period (2005–2007). The experiment involved two nutrient levels (based on the active agent equivalence principle): Level l: the NPK equivalent of 35 t ha^{-1} farmyard manure (FYM), applied in the form of FYM, FYM + mineral fertiliser or mineral fertiliser; Level 2: the NPK equivalent of 70 t ha^{-1} farmyard manure (FYM), applied in the form of FYM, FYM + mineral fertiliser or mineral fertiliser. The computerised growth analysis program elaborated by Hunt and Parsons (1974) was used to describe the effect of FYM and mineral fertiliser and to evaluate the results. This program fits functions to calculate the absolute growth rate (AGR), the relative growth rate (RGR), the net assimilation rate (NAR) and the leaf area ratio (LAR).

The Hunt–Parsons program fitted a third-degree function to the dynamics of total dry matter production and second- or third-degree functions to that of the leaf area growth. The highest mean values of AGR were obtained in treatments with the higher level of mineral fertiliser alone or mineral fertiliser + FYM when the weather was favourable (2.05–2.31 g $plant^{-1}$ day^{-1}), and in treatments with the lower quantity of mineral fertiliser alone or mineral fertiliser + FYM in the case of dry weather (1.73–1.74 g $plant^{-1}$ day^{-1}). In 2005 and 2006 the absolute growth rate gave a good characterisation of the various fertiliser effects, which exhibited high values with significant differences, while in 2007 lower AGR values were obtained and no fertiliser effects were observed. In the dry year (2007) the maximum values of NAR and LAR were higher in all the treatments than in the wetter years (except at the lower rate of mineral fertiliser alone). In the case of NAR, the results obtained with the functional method of growth analysis, based on function fitting, were easier to interpret than those obtained using the classical method.

It was concluded from the results that in long-term experiments the use of the functional method of growth analysis gave a more precise evaluation of the effects of fertiliser treatments and the year on the growth of maize in the vegetative growth stage and on the mean and maximum values of growth parameters.

Key words: maize, growth analysis, functional method, Hunt–Parsons model, AGR, RGR, NAR, LAR

Abbreviations: AGR, absolute growth rate; ALGR, absolute leaf area growth rate; LAR, leaf area ratio; NAR, net assimilation rate; RGR, relative growth rate; RLGR, relative leaf area growth rate

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Introduction

In the functional method of growth analysis, mathematical functions (generally polynomials or asymptotic functions) are fitted to measured data and the resulting growth functions are used to determine the momentary values of the individual parameters by means of differential calculus (Hunt, 1982; Causton and Venus, 1981). In general, parameters of the type $1/Y \times dY/dt$, Z/Y and $1/Z \times dY/dt$ are calculated, where Y is the dry mass of the whole plant, Z the leaf area and t the time. A detailed review of Hungarian papers dealing with the application of the functional method of growth analysis in crop production was published by Berzsenyi (2002). Mathematical functions fitted to observation data provide a better reflection of the reality observed by the scientist, and may thus be more valuable than the original data (Hunt, 1982).

The growth analysis program elaborated by Hunt and Parsons (1974) makes it possible to fit both lower (first- and second-degree) and higher (third-degree) polynomials. The most suitable function is chosen using the stepwise method. The application of the classical and functional methods of growth analysis, together with supplementary agronomic, ecological and physiological measurements, facilitates the multi-parametric evaluation of crop production experiments.

Using the classical and functional methods of growth analysis Berzsenyi (2010) studied the effect of N fertilizer on the dynamics of growth and growth parameters of three maize hybrids. Berzsenyi and Dang (2007) studied the effect of plant density on the growth of maize hybrids using the Richards function. Sugár and Berzsenyi (2010) analysed the growth dynamics and the yield of winter wheat grown at diverse nitrogen levels in a three-year field experiment.

Based on the results of over 40 years of long-term experiments set up on the principle of active agent equivalence, Berzsenyi and Győrffy (1997) reported that the yield was highest in treatments where half or all of the active agent content of FYM was replaced by NPK mineral fertiliser. According to Árendás and Csathó (2002) the efficiency of combined applications of FYM and mineral fertiliser is better than that of FYM alone, and approaches, but does not surpass, that of mineral fertiliser. Soil fertility in continuous maize can be improved without FYM through the regular application of mineral fertiliser. From the agronomic point of view, dry matter accumulation is the most important parameter, so in general this is used as an indicator of growth dynamics (Berzsenyi et al., 2007).

The aim of the present work was to apply the functional method of growth analysis to investigate the effect of various levels of FYM and mineral fertiliser on the growth of maize plants and the dynamics of growth parameters when grown in a monoculture in various years. The first results of this work were published by Micskei et al. (2010).

Materials and methods

Treatments

The long-term, small-plot experiment was set up by Béla Győrffy on partially eroded chernozem soil with forest residues in the experimental nursery of the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár in 1959. The experiment included the following treatments: (1) Control; (2) 35 t ha^{-1} FYM; (3) 17.5 t ha^{-1} FYM + $N_{1/2}P_{1/2}K_{1/2}$ mineral fertiliser; (4) $N_1P_1K_1$ mineral fertiliser; (5) 70 t ha^{-1} FYM; (6) 35 t ha^{-1} FYM + $N_1P_1K_1$ mineral fertiliser; (7) $N_2P_2K_2$ mineral fertiliser (hereafter: Treatments 1–7). The annual active agent quantities applied (kg ha^{-1}) were N 66, P_2O_5 38, K_2O 75 in Treatments 2–4 and N 132, P_2O_5 76, K_2O 150 in Treatments 5–7. The FYM and the P and K fertiliser were applied in autumn every 4 years, most recently in 2006. The 4-year N dose was distributed in equal portions each year. The FAO 380 Martonvásár maize hybrid Norma SC was sown between April 17th and 30th with row and plant distances of 70 × 20 cm. The experiment was laid out in a Latin square design with 7 replications, with a plot size of 80 m^2.

Year effect

The rainfall and temperature data of the three years exhibited substantial differences, compared to each other and to the 30-year mean. The weather was very favourable for maize in 2005 as regards both rainfall and temperature at sowing, thus promoting ideal emergence. In 2006 only half the usual rainfall quantity was recorded during the sowing period, but the sum for the whole year was around average. The weather in 2007 was extremely hot and dry, with mean temperatures 2°C higher than the long-term mean for every month. The rainfall quantity during the vegetation period (Apr.–Sep.) in the three years was as follows: 526 mm in 2005, 342 mm in 2006, 315 mm in 2007. The mean annual temperature was 9.8°C in 2005, 10.9°C in 2006 and 12.3°C in 2007.

Sampling and measurements

Both the destructive (direct) and indirect methods of growth analysis were applied. Sampling was begun when the maize plants reached the 4-leaf stage of development (22–37 days after sowing) and was continued until physiological maturity. For the destructive analysis three plants were cut at ground level from each replication of each treatment every 14 days. The plants were divided into the following organs: green leaf-blade, stalk + leaf sheath, tassel, ear husk, ear stalk, ear and kernels. Measurements were made on the fresh mass of the plant organs, the leaf area and leaf number, and the dry mass of the separate organs after drying at 105°C for 48–72 h. For the indirect analysis, the leaf area above the ear and the chlorophyll content were recorded for the plant stand.

Growth analysis with the Hunt–Parsons program

The characterisation of FYM and mineral fertiliser effects and the evaluation of the results were achieved with the functional method of growth analysis, which fits mathematical functions to the primary data and uses differential calculus to determine the momentary values of various parameters from the resulting growth functions. The Hunt-Parsons growth analysis program applies the stepwise regression method to fit first-, second- or third-degree polynomials to the whole plant dry matter (Y) and the total leaf area (Z) as a function of time (t), also providing the standard error and the 95% confidence intervals for all the sampling periods (Berzsenyi, 2002). The program carries out analysis of variance and gives the equations of the polynomials. It then calculates the observed and fitted values of the variables Y and Z and of ln Y and ln Z for every value of t. The program determines the absolute growth rates (AGR, ALGR), the relative growth rates (RGR, RLGR), the net assimilation rate (NAR) and the leaf area ratio (LAR). The results obtained with the Hunt-Parsons program were then compared on the basis of the mean and maximum values and the dynamics of the parameters.

Results and discussion

Dynamics and absolute growth rate of dry matter production in maize plants, based on the Hunt–Parsons model

The Hunt-Parsons program used a third-degree function to characterise the dynamics of total dry matter production in every treatment in all the years, with the exception of Treatment 2 in 2006, where a second-degree function was fitted. The shape of the growth curves was similar in all three years, but a more detailed analysis revealed important differences, demonstrating the effects of the various fertilisation treatments and years on plant growth (Fig. 1). In 2005 and 2006 the effects of the various treatments were quite distinct, while in 2007 the values of dry matter production in the individual treatments varied over a much narrower range. In 2005, a favourable year for maize, the significantly lowest dry matter production (145 g day^{-1}) was recorded in the control and the highest (308 g day^{-1}) in Treatment 7 ($N_2P_2K_2$), with intermediate values in Treatments 3, 4 and 5 (243–267 g day^{-1}). In 2006 the lowest dry matter production (150 g day^{-1}) was again obtained in the control and the highest in Treatment 7 (267 g day^{-1}). However, the maximum value recorded in Treatment 5 (197 g day^{-1}) was lower than that observed in Treatments 2 and 3 (213 and 223 g day^{-1}, respectively), while there was no significant difference between Treatments 4 and 6 (244 and 249 g day^{-1}, respectively). In 2007, when the weather was unfavourable for maize, the highest dry matter production (200–212 g day^{-1}) was obtained in treatments given only FYM (Treatments 2 and 5) and in Treatment 3, given the lower rate of FYM + mineral fertiliser. It could thus be concluded that in dry years the effect of FYM surpasses that of mineral fertiliser, as confirmed by the fact that the lowest values of dry matter production were found in Treatment 6 and in the control (178 and 181 g day^{-1}, respectively), while equal results were obtained in Treatments 4 and 7 (185 g day^{-1}). It can be seen from the shape of the curves that in 2005, thanks to the optimum rainfall supplies, the curve did not reach a maximum at the end of the growth period, indicating that growth was still continuing. In the drier year of 2006 the incorporation of dry matter exhibited a pronounced decline at the end of the growth period, having previously reached a maximum in all the fertiliser treatments. In Treatment 2 plant growth ceased 10–12 days earlier. 2007 was a dry year, so the maximum values of dry matter production were low in all the treatments and were reached two weeks earlier than in the more favourable years. In Treatments 3 and 4, however, the values rose again at the end of the vegetation period.

Fertiliser-dependent changes in the dynamics of dry matter production were clearly reflected by the absolute growth rate (Fig. 1), which gradually rose to a maximum, after which it declined. The absolute growth rates obtained in the different treatments varied over a narrower range in the dry year. In 2005 and 2006 the AGR curve in the control was clearly distinct from those of the other

fertiliser treatments and exhibited the lowest values, while in 2007 this difference could only be detected up to heading. In favourable years the values of AGR, like the dynamics of dry matter production, were highest in Treatments 6 and 7 (the higher rate of mineral fertiliser or mineral fertiliser + FYM), while in the dry year the highest values were obtained at the lower rate of these forms (Treatments 2 and 3).

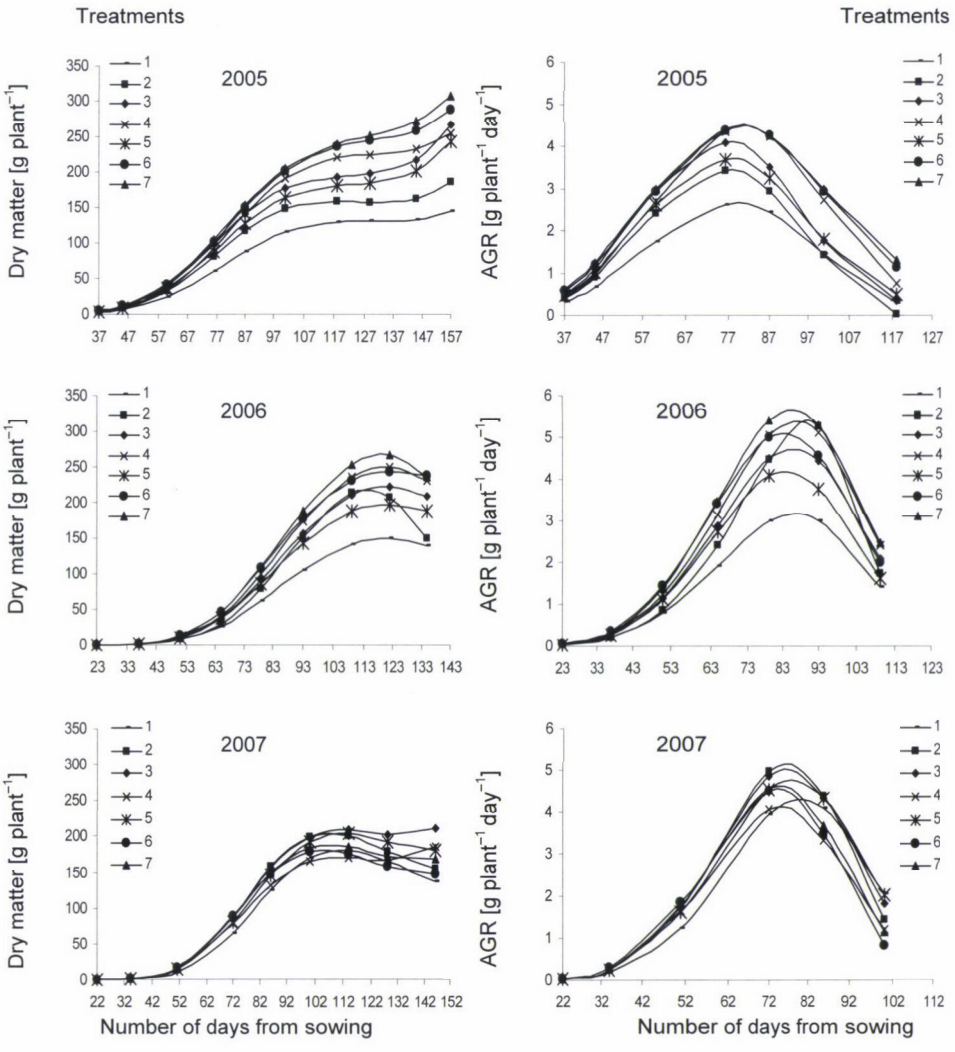

Fig. 1. Effect of fertilisation and years (2005–2007) on the dynamics of total dry matter production and absolute growth rate (AGR) of maize plants based on the Hunt-Parsons program. Treatments: 1. Control; 2. 35 t ha^{-1} FYM; 3. 17.5 t ha^{-1} FYM + $N_{1/2}P_{1/2}K_{1/2}$; 4. $N_1P_1K_1$; 5. 70 t ha^{-1} FYM; 6. 35 t ha^{-1} FYM + $N_1P_1K_1$; 7. $N_2P_2K_2$.

After flowering, the AGR and RGR values exhibited considerable variability, so the mean values of these parameters were calculated up to 72–79 days after sowing (Table 1). The mean value of AGR was highest in 2005, averaged over the treatments (1.98 g plant^{-1} day^{-1}), and lowest in 2007 (1.61 g plant^{-1} day^{-1}). In 2005 and 2006 the absolute growth rate clearly reflected the fertiliser effects, which exhibited significant differences and high values, while in 2007 lower AGR values were obtained and no fertiliser effect was observed. The mean values of AGR were highest at the higher rate of mineral fertiliser or mineral fertiliser + FYM in favourable years (2.05 and 2.31 g plant^{-1} day^{-1}) and at the lower rate of these treatments in the dry year (1.74 g plant^{-1} day^{-1}). In all the fertiliser treatments the maximum AGR values were the lowest in 2005. Averaged over the treatments, the value of RGR was the lowest in 2005 (0.0763 g g^{-1} day^{-1}) and higher in 2006 and 2007 (0.1083 and 0.1160 g g^{-1} day^{-1}, respectively). These results confirm that the relative growth rate calculated for the whole plant is not a sufficiently sensitive parameter to give a precise picture of fertiliser and year effects.

Seasonal dynamics of the leaf area and absolute growth rate of the leaf area of maize plants, based on the Hunt–Parsons model

The Hunt–Parsons program fitted a second-degree function to the dynamics of leaf area growth in 2005, second- and third-degree functions in 2006 and a third-degree function in 2007. In 2005 and 2006 the effects of the seven fertiliser treatments could be clearly distinguished on the basis of the seasonal dynamics of the leaf area (as in the case of dry matter dynamics), while these differences were less pronounced in 2007 (Fig. 2). In 2005 the maximum leaf area was the lowest in Treatment 2 (3716 cm^2) and the highest in Treatment 6 (5990 cm^2). The difference between the maximum leaf areas increased in 2006, with the lowest value in the control (3353 cm^2) and the highest in Treatment 7 (5845 cm^2). Treatment 7 also gave the highest maximum leaf area in 2007 (5690 cm^2), while the smallest value was recorded in Treatment 5 (4486 cm^2).

Table 1

Effect of fertilisation treatments on the mean and maximum values of absolute growth rate for total dry matter (AGR; g plant^{-1} day^{-1}) and the mean values of relative growth rate (RGR; g g^{-1} day^{-1}) during the vegetative period (Martonvásár, 2005–2007)

Treatment	AGR						RGR		
	2005		2006		2007		2005	2006	2007
	Mean	Max.	Mean	Max.	Mean	Max.	Mean		
1	1.33	2.60	1.19	3.00	1.34	4.09	0.0757	0.1048	0.1169
2	1.78	3.42	1.60	5.25	1.74	4.97	0.0790	0.1045	0.1197
3	2.15	4.10	1.77	4.51	1.73	4.87	0.0788	0.1086	0.1188
4	2.11	4.33	1.95	5.11	1.49	4.06	0.0802	0.1100	0.1155
5	1.94	3.68	1.65	4.08	1.60	4.55	0.0762	0.1103	0.1141
6	2.27	4.39	2.05	5.00	1.67	4.51	0.0729	0.1094	0.1139
7	2.31	4.41	2.13	5.42	1.69	4.57	0.0715	0.1106	0.1134

For treatments, see Figure 1

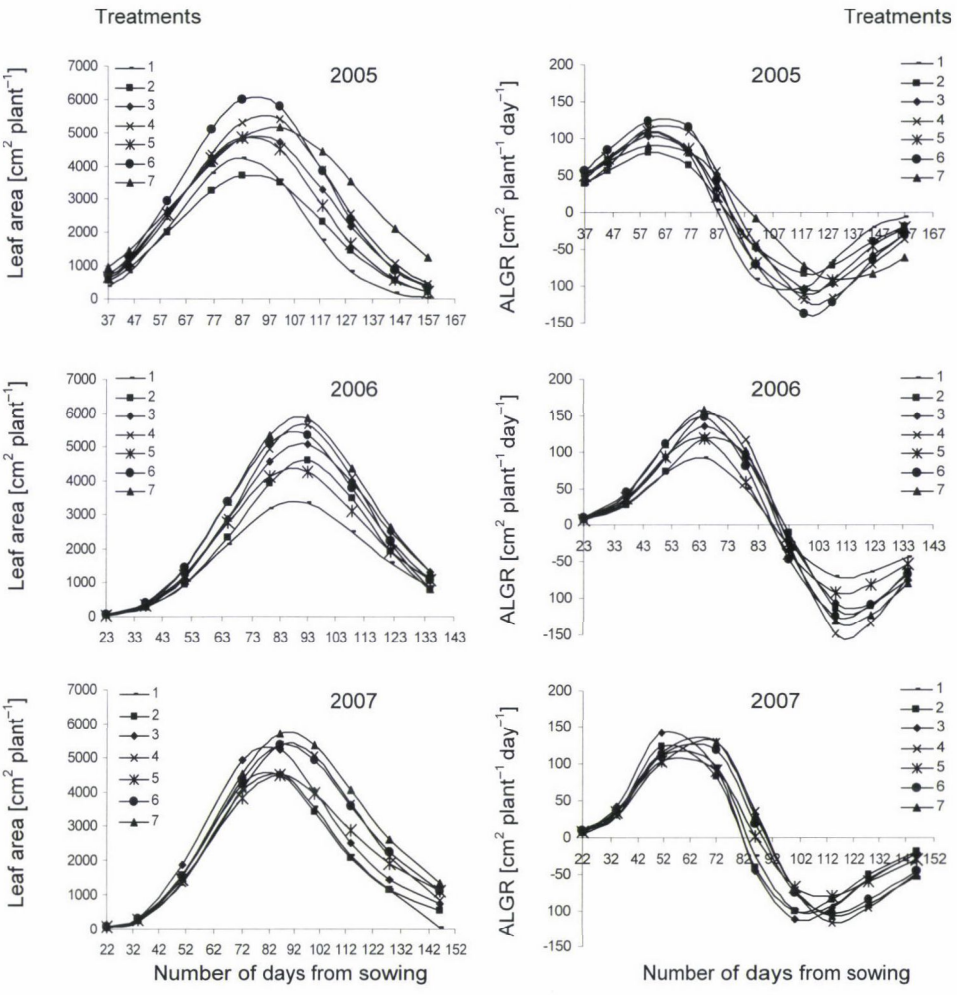

Fig. 2. Effect of fertilisation and years (2005–2007) on the seasonal dynamics of the leaf area and the absolute growth rate (ALGR) of maize plants based on the Hunt–Parsons program. For treatments, see Figure 1

Leaf area growth was characterised by an increase in the growth rate (ALGR) up to a maximum, followed by a gradual decrease right up to the end of growth. This was followed by a steep decline in the leaf area (withering). The maximum and mean values of ALGR gave a good reflection of both fertiliser and year effects (Table 2).

Table 2

Effect of fertilisation treatments on the mean and maximum values of absolute growth rate of the leaf area (ALGR; cm^2 $plant^{-1}$ day^{-1}) and the mean values of relative growth rate (RLGR; cm^2 cm^{-2} day^{-1}) during the leaf growth period (Martonvásár, 2005–2007)

Treatment	ALGR						RLGR		
	2005		2006		2007		2005	2006	2007
	Mean	Max.	Mean	Max.	Mean	Max.	Mean		
1	58.1	107.8	49.2	91.5	60.6	111.7	0.0505	0.0795	0.1093
2	52.0	81.5	64.3	118.3	61.6	122.7	0.0394	0.0776	0.1072
3	68.5	102.8	72.8	135.7	71.7	142.4	0.0388	0.0795	0.1050
4	78.9	113.7	80.3	150.6	67.6	130.3	0.0447	0.0799	0.0949
5	67.9	109.4	63.0	118.7	59.2	102.8	0.0415	0.0811	0.0946
6	84.8	123.9	78.7	148.2	69.5	118.8	0.0436	0.0791	0.0929
7	68.6	89.9	84.3	158.2	72.9	129.4	0.0328	0.0808	0.0933

For treatments, see Figure 1

The mean value of ALGR was smallest in Treatment 2 and greatest in Treatment 6 in 2005, while in 2006 and 2007 the control treatment gave the lowest value and Treatment 7 the highest. Averaged over the treatments, the mean value of ALGR was the lowest in 2007 (66 cm^2 $plant^{-1}$ day^{-1}), higher in 2005 (68 cm^2 $plant^{-1}$ day^{-1}) and the highest in 2006 (70 cm^2 $plant^{-1}$ day^{-1}), while the maximum ALGR was the lowest in 2005 (105 cm^2 $plant^{-1}$ day^{-1}), higher in 2007 (123 cm^2 $plant^{-1}$ day^{-1}) and the highest in 2006 (132 cm^2 $plant^{-1}$ day^{-1}). Averaged over the treatments the value of RLGR was the lowest in 2005 (0.0416 cm^2 cm^{-2} day^{-1}), higher in 2006 (0.0796 cm^2 cm^{-2} day^{-1}) and the highest in 2007 (0.0996 cm^2 cm^{-2} day^{-1}).

Seasonal dynamics of net assimilation rate and leaf area ratio of maize plants, based on the Hunt–Parsons model

The effect of the fertiliser treatments and the year on the seasonal dynamics of the net assimilation rate and leaf area ratio of maize plants is illustrated in Figure 3. In the case of NAR, an increasing tendency could be observed during the initial development phase, followed by a gradual decline after the maximum was reached. In 2005 the maximum value of NAR was reached on the 45th day after sowing in the control and around the 58th day in all the other treatments. In 2006 the momentary values of NAR were approximately the same in all the treatments between days 45 and 95, with the exception of Treatment 2, where the NAR value rose gradually to the 93rd day and then suddenly declined. In 2007 the NAR dynamics of the control and Treatment 3 were similar to that of the control treatment in 2006, while in Treatments 4, 6 and 7 the NAR values increased up to day 52 and in Treatments 2 and 5 until day 70.

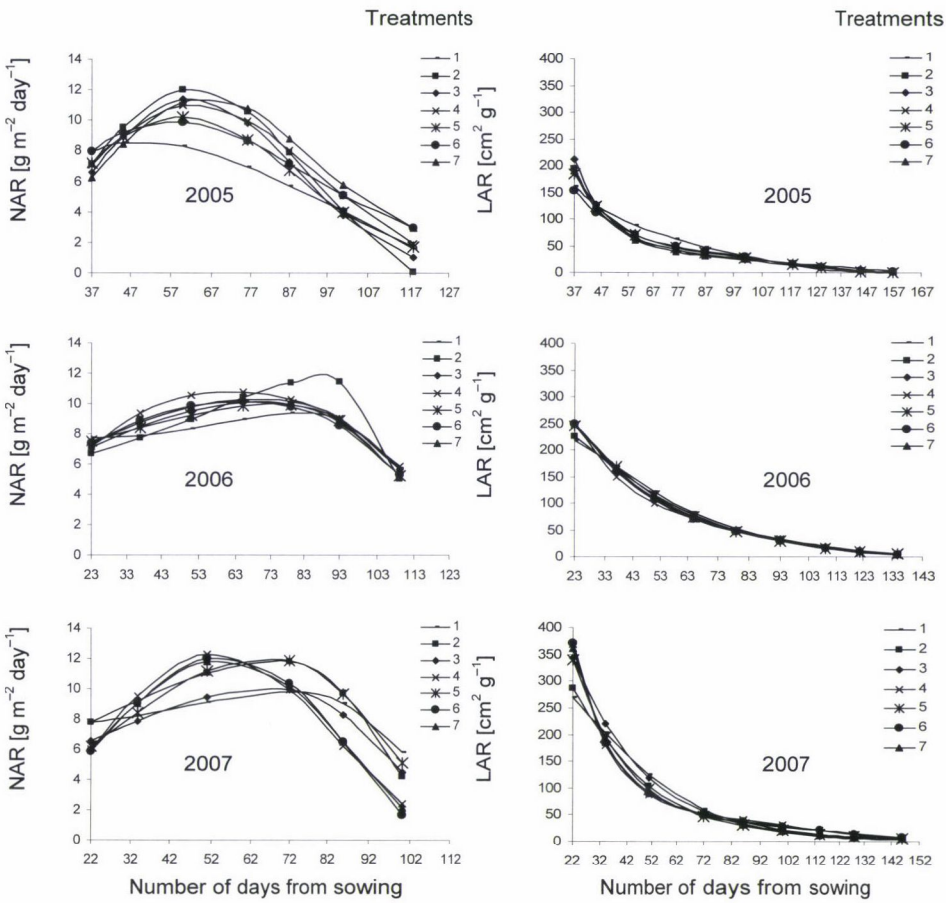

Fig. 3. Effect of fertilisation treatments and years (2005–2007) on the seasonal dynamics of the net assimilation rate (NAR) and leaf area ratio (LAR) of maize plants based on the Hunt–Parsons program. For treatments, see Figure 1

After an initial maximum (measured at the first sampling date every year), the seasonal dynamics of LAR exhibited a gradual decrease right up to the end of the vegetation period. Irrespective of the year, the control gave the lowest LAR value at the first sampling, while from the second sampling onwards the values in the control were higher than those in the other treatments. There were significant differences between the NAR and LAR values calculated by the Hunt-Parsons model in terms of both fertiliser treatment and year (Table 3). Using classical growth analysis significant differences were only obtained between the years, and not between the fertiliser treatments. In the dry year the model revealed significant differences between the mean LAR values. In all the treatments the maximum LAR values were the lowest in 2005 (153–211 cm^2 g^{-1}), higher in 2006 (218–249 cm^2 g^{-1}) and the highest in 2007 (270–371 cm^2 g^{-1}).

Table 3

Effect of fertilisation treatments on the mean and maximum values of net assimilation rate (NAR; g m^{-2} day^{-1}) and leaf area ratio (LAR; cm^2 g^{-1}) during the vegetative period (Martonvásár, 2005–2007)

Treatment	NAR						LAR					
	2005		2006		2007		2005		2006		2007	
	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.
1	7.47	8.49	8.47	9.38	8.79	9.80	97.2	161.6	128.1	217.7	139.2	270.2
2	9.41	11.99	9.05	11.44	9.90	11.76	89.8	194.4	126.8	224.9	131.8	287.8
3	8.80	11.36	9.01	10.04	8.41	9.91	96.2	211.0	129.2	248.8	154.8	344.3
4	9.00	10.97	9.63	10.79	8.80	12.29	94.0	190.6	123.5	247.9	145.6	368.0
5	8.35	10.24	8.99	9.90	9.47	11.89	92.6	184.0	129.7	244.6	142.1	339.8
6	8.56	9.90	9.22	10.17	8.75	12.01	84.8	152.7	126.2	248.2	146.4	370.9
7	9.09	11.19	9.25	10.30	8.68	11.76	88.0	189.8	128.0	248.7	145.4	361.5

For treatments, see Figure 1

When the functional method of growth analysis was applied, significant differences were detected between the NAR values in terms of both years and fertiliser treatments, whereas only the year effect was significant in the case of the classical method (Micskei et al., 2010). Averaged over the treatments, the mean value of NAR was lowest in 2005 (8.67 g m^{-2} day^{-1}), the highest in 2006 (9.09 g m^{-2} day^{-1}) and somewhat lower in 2007 (8.97 g m^{-2} day^{-1}), while the maximum value was approximately the same in 2005 and 2006 (10.59 and 10.29 g m^{-2} day^{-1}, respectively) and significantly higher in 2007 (11.35 g m^{-2} day^{-1}). In the wet years the mean NAR values were lowest in the control (7.47 and 8.47 g m^{-2} day^{-1}) and highest in Treatment 2 in 2005 (9.41 g m^{-2} day^{-1}) and in Treatment 4 in 2006 (9.63 g m^{-2} day^{-1}). In the dry year this value was smallest in Treatment 3 (8.41 g m^{-2} day^{-1}) and greatest in Treatment 2 (9.90 g m^{-2} day^{-1}). In the unfavourable year the effect of the fertiliser treatments was less pronounced.

Averaged over the treatments, the maximum values of LAR were the lowest in 2005 (92 cm^2 g^{-1}), rising in 2006 (127 cm^2 g^{-1}) and giving the significantly highest values in 2007 (144 cm^2 g^{-1}). The mean LAR value was smallest in Treatment 6 and greatest in the control in 2005, lowest in Treatment 4 and highest in Treatments 3 and 5 in 2006, and smallest in Treatment 2 and greatest in Treatment 3 in 2007. In the dry, hot year of 2007 the maximum value of NAR and both the mean and maximum values of LAR were higher in all the treatments than in the cooler, wetter year of 2006.

Conclusions

The Hunt-Parsons program fitted a third-degree function to the dynamics of total dry matter production and second- or third-degree functions to that of leaf area growth. Averaged over the treatments, the mean value of AGR was the highest in 2005 (1.98 g $plant^{-1}$ day^{-1}) and lowest in 2007 (1.61 g $plant^{-1}$ day^{-1}). This parameter clearly distinguished between the fertiliser treatments in 2005 and 2006, when the values were high and significantly different, while in 2007 lower values were recorded and no fertiliser effect was detected.

Significant differences were observed between the NAR values in response to both years and fertiliser treatments when the functional method of growth analysis was applied, while only the year effect was significant according to the classical method. In the dry year the maximum values of NAR and LAR were higher in all the treatments than in the cooler, wetter years.

The functional method of growth analysis, based on the fitting of functions, gave a better interpretation of the results than the classical method in the case of NAR and RGR, but for LAR fitting functions did not give a clearer picture of the real situation than that obtained with the classical method. The dynamics of maize growth in various treatments and years could best be described using the AGR parameter. Using the Hunt–Parsons growth analysis program significant differences were obtained between the fertiliser treatments and the years during various stages in the growth of maize plants, suggesting that the program could be widely applied in crop production research.

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PHOTOSYNTHESIS IN THE 7H ASAKAZE KOMUGI/MANAS WHEAT/BARLEY ADDITION LINE DURING SALT STRESS

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The photosynthetic responses induced by NaCl were investigated in the 7H Asakaze komugi/Manas wheat/barley addition line developed in the Agricultural Research Institute, Martonvásár, Hungary, in the wheat (*Triticum aestivum* L.) cv. Asakaze komugi (Akom) and wheat line Martonvásári 9 kr1 (Mv9kr1) and in the barley (*Hordeum vulgare* L.) cv. Manas. An increase in the NaCl concentration of the nutrient solution to 200 mmol L^{-1} resulted in considerable stomatal closure and a decreased net CO_2 assimilation rate (A) in the wheat genotypes, while the changes in these parameters were less significant for barley and the 7H addition line. Parallel with this, a relatively high non-stomatal limitation (L_m) of A was observed in wheat genotypes, which was not significant in Manas or the wheat-barley addition line at this level of salt stress. At severe stress (300 mM L^{-1} NaCl concentration) A and stomatal conductance were strongly inhibited in all the genotypes examined; however, L_m was less significant in the addition line and its parental wheat genotype. These preliminary results suggest that the 7H Akom/Manas addition line might be a good candidate for improving the salt tolerance of wheat in the future, and encourage further detailed physiological analysis of this addition line.

Key words: salt stress, wheat, barley, photosynthesis, wheat/barley addition line

Abbreviations: A, net CO_2 assimilation rate; Akom, Asekaze komugi; 7H add, 7H Asakaze komugi/Manas addition line; g_s, stomatal conductance; L_m, non-stomatal limitation, L_s, stomatal limitation; RWC, relative water content.

Introduction

Salt stress is considered to be an important environmental factor, limiting plant growth and productivity in today's agriculture (Boyer, 1982; Kingsbury et al., 1984; Belkhodja et al., 1999; Allakhverdiev et al., 2000), especially in arid or semi-arid regions (Siler et al., 2007) or irrigated areas (Szabolcs, 1994). In crop plants several life processes are particularly sensitive to soil and water salinity, since high salt concentration has a threefold effect: it limits water availability to the roots (osmotic stress); it can cause ionic stress when the salt is

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taken up by plants; and it also influences nutrient uptake and translocation (Bagci, 2003; Siler et al., 2007). Stomatal behaviour and photosynthesis are some of the most important processes suppressed by abiotic stress factors like drought, heat and salt (El-Shintinawy, 2000; Hoffmann et al., 2006). The decrease in photosynthesis during salt stress can be attributed to the reduced activity of primary photochemical processes (Kaiser et al., 1983) and to the inhibition of carbon dioxide fixation and assimilation (Flowers et al., 1977; Munns and Termaat, 1986; Khavari-Nejad and Mostofi, 1998; El-Shintinawy, 2000; Siler et al., 2007). The reduction in the CO_2 assimilation rate (A) caused by salt stress is mainly attributed to the decrease in stomatal conductance (g_s) (Centritto et al., 2003) and/or to the non-stomatal limitation (Bongi and Loreto, 1989; Brugnoli and Björkman, 1992; Delfine et al., 1999; Centritto et al., 2003) related to the processes of carbon fixation, such as the diffusion of CO_2 from the intercellular spaces to the chloroplasts, and to other important metabolic factors. According to some studies, salt stress has no effect on Photosystem II or primary photochemical processes (Robinson et al., 1983; Brugnoli and Björkmann, 1992; Morales et al., 1992), but the inhibition of carbon dioxide fixation is well known (Flowers et al., 1977; Munns and Termaat, 1986; Khavari-Nejad and Mostofi, 1998; El-Shintinawy, 2000; Siler et al., 2007). In any case, to maintain the photosynthetic rate at a promising level during salt stress may be an advantageous feature, since it makes adequate growth and biomass production possible. Furthermore, the estimation of net assimilation rate might facilitate discrimination between salt-tolerant and non-tolerant cultivars of barley (Belkhodja et al., 1999). As described above, the effects of salt stress and salt tolerance have been widely examined, and a large body of data is available on the salt tolerance of wheat and barley and on the effects of salinity on these plants (Belkhodja et al., 1999; El-Shintinawy, 2000; Bagci, 2003; Veselov et al., 2009).

The development of salt-tolerant crops bears significant agricultural and economic importance, as soil salinity decreases the growth, dry matter production and yield of cultivated plants. To date, various approaches have been used to produce stress-tolerant plants (Stiller et al., 2008). In the case of wheat these include chromosome-mediated gene transfer from stress-tolerant donor species to wheat. Barley is a potential gene source for wheat improvement, because of its generally good stress tolerance (Cattivelli et al., 2002; Molnár et al., 2007), earliness (Murai et al., 1997) and nutritional parameters (Islam and Shepherd, 1990). The introgressive hybridization of barley to wheat makes it possible to transfer useful salt tolerance characteristics, as barley is regarded as being more salt-tolerant than bread wheat (Colmer et al., 2005; 2006). Furthermore, the addition of the 7H barley chromosome pair into wheat resulted in a modified Na^+/K^+ ratio in the leaves with lower Na^+ content, indicating a positive interaction between the individual *H. vulgare* chromosomes and the bread wheat genome to enhance Na^+ exclusion (Colmer et al., 2006). This suggests that the 7H addition line might have better salt tolerance.

In the present paper the photosynthetic responses to salt stress in the parental winter wheat (Asakaze komugi) and barley (Manas) cultivars were compared with those of a 7H Asakaze komugi–Manas addition line and with Mv9kr1 winter wheat in order to indicate whether the newly developed addition line had better tolerance to moderate salt stress than wheat, making it suitable for improving the salt tolerance of wheat.

Materials and methods

Plant materials

Salt stress was applied to the 7H wheat/barley addition line (7H add) developed from the Asakaze komugi × Manas hybrid produced in Martonvásár (Molnár-Láng et al., 2000; 2005; 2007), together with the parental lines, wheat (*Triticum aestivum* L.) cultivar Asakaze komugi (Akom) and barley (*Hordeum vulgare* L.) cv. Manas. Mv9 kr1 was also used in this experiment as this line originates from a Martonvásári wheat cultivar. The crossability alleles were transferred from the cultivar Chinese Spring into cv. Martonvásári 9, so the Martonvásári 9 kr1 (Mv9 kr1) line is frequently used in wide crosses in Martonvásár (Molnár-Láng et al., 1996). Salt stress was induced by increasing the NaCl concentration of the hydroculture medium.

Hydroculture system

Seeds germinated under laboratory conditions were grown in pots containing 1500 mL of half-strength modified Hoagland nutrient solution (Nagy and Galiba, 1995; Molnár et al., 2004) in growth chambers under normal CO_2 concentration at 20/25°C. The light intensity for growth was 200 μmol m^{-2} s^{-1} with a 12/12 h photoperiod. For each genotype, 20 plants (five plants/pot) were grown. Salt stress was induced after 4 weeks by applying sodium chloride (NaCl, Sigma, St Louis, MO) in 7-day cycles at increasing concentrations of 100, 200 and 300 mmol L^{-1}. The solution was renewed twice a week. Measurements were done after 7-day treatments with 100, 200 and 300 mmol L^{-1} NaCl and after 2 and 7 days of regeneration without NaCl.

Determination of relative water content (RWC)

The water status of the plants was traced by determining the relative water content (RWC) according to the following equation:

$$RWC = (FW - DW) \times 100 / (SW - DW)$$

where FW is the fresh weight, SW the water-saturated weight and DW the dry weight after drying for 12 h at 105°C.

Gas exchange measurements

The CO_2 assimilation of intact leaves was measured with an infrared gas analyser (GFS-3000 gas exchange system, Walz, Effeltrich, Germany). The light used to induce photosynthesis was provided by a LED array (3055 FL, Walz, Effeltrich, Germany). The net CO_2 assimilation rate (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were calculated in the light-saturated state of photosynthesis (Dulai et al., 2006) using the equations reported by von Caemmerer and Farquhar (1981). The responses of A to changes in the ambient CO_2 concentration were measured between 3 and 1000 ppm CO_2 at 1000 μmol m^{-2} s^{-1} light intensity. Stomatal (L_s) and non-stomatal (L_m) limitation were determined on the basis of the A *vs.* C_i curves, as described by Lawlor (2002).

Statistical analysis

The results are the means of 5 measurements per treatment. Student t-tests were performed using MS Excel (Microsoft Corporation, Seattle, USA). Differences between the results are described as being significant where $P \leq 0.05$, and not significant where $P > 0.05$.

Results

Effects of salt stress on water content and stomatal conductance

The plants were grown under optimal conditions for 4 weeks. Salt treatment was started in the fifth week and the responses of the plants were studied. In all the genotypes examined the water loss was more or less continuous, in parallel with the increasing salt concentration (Fig. 1A). A significant decrease in RWC was recorded even at 100 mmol L^{-1} NaCl in the case of 7H add, but there was little further water loss in this line. The greatest decline was observed for barley during salt treatment at 300 mmol L^{-1}. Although the RWC also decreased in the wheat genotypes, it was moderate even at the end of the salt treatment and was statistically significant compared with the RWC values of barley ($P \leq 0.01$). During regeneration, Manas, 7H add and Mv9 kr1 recovered their original water contents by the end of the second day and full regeneration was observed in all the lines by the 7th day. The initially high stomatal conductance (g_s), which is proportional to the closure of the stomatal aperture, decreased in all the genotypes in parallel with water loss (Fig. 1B). The initially highest g_s and the strongest stomatal closure were detected for Akom wheat even at weak stress (100 mmol L^{-1} NaCl). In contrast to the wheat genotypes, the decrease in g_s was moderate in barley at 200 mmol L^{-1} NaCl and the loss in stomatal conductance was less than 50% at 300 mmol L^{-1} NaCl, as in 7H add. Although the difference in g_s was not considerable between the genotypes at severe salt stress, this was the result of the loss of more than 70% of the original activity in the case of wheat cv. Akom, but was less than 40% for 7H add.

Changes of net photosynthetic CO_2 fixation during salt stress

CO_2 gas analysis revealed a moderately higher net assimilation rate (A) in Mv9 kr1 than in the other genotypes (Fig. 1C) for untreated plants, and this difference was statistically significant ($P \leq 0.01$). Like g_s, A dropped sharply in Akom in parallel with the increase in salt concentration and this decrease was significant even at 100 mmol L^{-1} NaCl ($P \leq 0.01$) compared to the control, while A was practically unchanged in barley and 7H add. When the salt concentration reached 200 mmol L^{-1}, A was strongly inhibited in the wheat lines, leading to a loss of more than 40% of the original activity, while in barley and 7H add it remained high, with less than 12% decrease in the original activity levels. The difference in A between the two groups was also significant ($P \leq 0.01$) at this salinity level. These results suggest that the limitation of photosynthesis in the wheat cultivars could be attributed to different factors than in barley and 7H add at this moderate stress level. This is reflected in the fact that non-stomatal limitation (L_m) was relatively high in wheat (Fig. 2B), but negligible for Manas and 7H add. L_s was high in all the genotypes examined. Furthermore, at 300 mmol L^{-1} NaCl L_m was lower ($P \leq 0.05$) in Akom and 7H add than in barley and Mv9 kr1. The changes in non-stomatal limitation in 7H add followed those recorded for Manas at an intermediate salinity level (200 mmol L^{-1}, where L_m was low); on the other hand, at a severe salt stress level (300 mmol L^{-1}) they were similar to those of Akom, which had lower values of L_m than Manas.

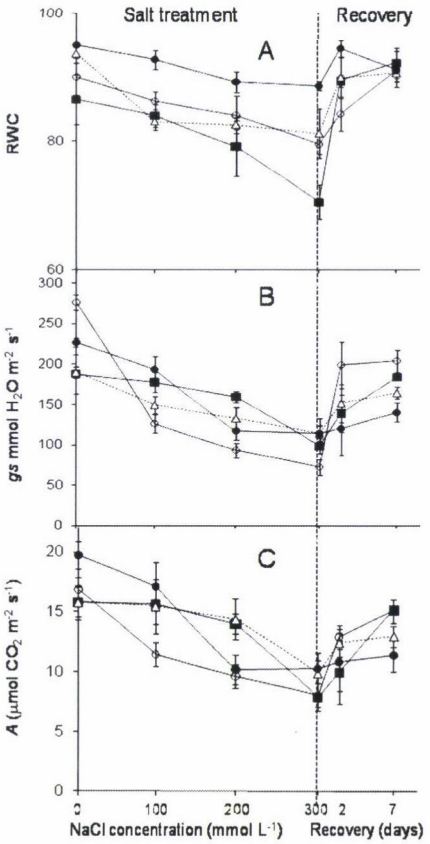

Fig. 1. Effects of increasing NaCl concentration, followed by 7 days recovery on (A) relative water content (RWC), (B) stomatal conductance (g_s) and (C) net CO_2 assimilation rate (A) in wheat (continuous lines), barley (continuous line) and the 7H Akom-Manas addition line (dotted line). (•) Mv9 kr1, (○) Akom V, (■) Manas, (□) 7H Akom-Manas addition line. The results are the means of data from five plants per treatment

Discussion

In this study the photosynthetic performance of the newly developed 7H Akom/Manas addition line (7H add) was examined at various salt concentrations. In this preliminary report, photosynthetic and physiological responses to salinity were compared with those of the parental genotypes and with Mv9 kr1 wheat. The aim was to clarify whether the tolerance of barley cv. Manas to moderate salt stress manifests itself in this addition line, and whether it could be suitable for improving the salt tolerance of bread wheat. These examinations were encouraged by earlier findings suggesting that the addition of the 7H chromosome pair improved salt tolerance (Colmer el al., 2006).

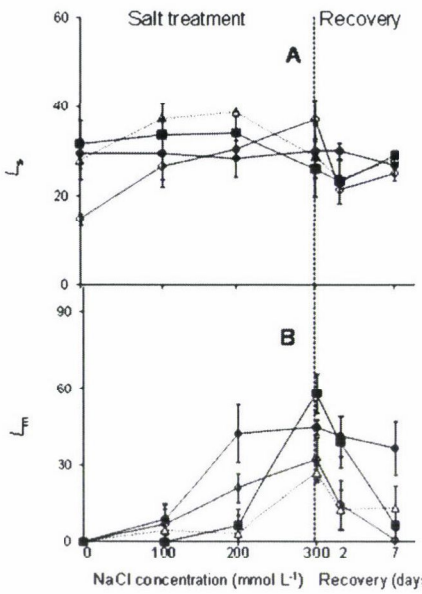

Fig. 2. Effects of increasing NaCl concentration, followed by 7 days recovery on (A) stomatal limitation (L_s) and (B) non-stomatal limitation (L_m) in wheat (continuous lines), barley (continuous line) and the 7H Akom-Manas addition line (dotted line). (•) Mv9 kr1, (○) Akom V, (■) Manas, (□) 7H Akom-Manas addition line. The results are the means of five plants per treatment

Several physiological processes, including changes in ion balance, water status, photosynthesis, stomatal behaviour, etc., are modified during salt stress (Flowers et al., 1977; Munns and Termaat, 1986). As salt stress not only has an ionic effect but also leads to osmotic stress, the water balance of plants is changed during salt stress, as a result of which the RWC of the leaves decreases. In most cases stomatal closure can be observed, parallel with which the stomatal conductance to water vapour (g_s) decreases (Centritto et al., 2003). Salt stress caused a faster reduction in RWC in 7H add at 100 mmol L^{-1} NaCl than in wheat and barley cultivars, but in the case of severe salinity it remained above 80%, while the RWC of barley was considerably decreased (Fig. 1A). Parallel with the slow decrease in water content the initially high stomatal conductance (g_s) dropped sharply in Akom (Fig. 1B), and decreased continuously in Mv9 kr1. In this connection, stomatal closure is well known to be the most efficient way of reducing transpirational water loss (Cornic, 2000). Although the decrease in g_s was only 30% for 7H add, the water content remained at a level similar to that of Akom, where g_s was only a fraction of the control value at severe stress. These results show that 7H add was able to maintain its water status more successfully than barley without pronounced stomatal closure.

As the stomata play an important role in the regulation of transpirational water loss during salt stress, the primary physiological effect of salinity is the inhibition of photosynthetic CO_2 fixation, partly by means of stomatal closure (Centritto et al., 2003). The most rapid stomatal closure was detected at a weak salt stress of 100 mmol L^{-1} NaCl in wheat cv. Akom, where stomatal closure was not significant for Manas. When other factors do not influence carboxylation processes, this decrease in stomatal conductance (g_s), which may restrict the diffusion of CO_2 into the leaves, has been reported to lead to a modification of the intercellular CO_2 level, resulting in a decrease in photosynthetic CO_2 fixation (Flexas and Medrano, 2002). A decreased substantially as g_s fell in Akom and it was also low in Mv9 kr1 at moderate stress (200 mmol L^{-1} NaCl). At this level of salinity, however, A and g_s only decreased slightly in barley and 7H add, with residual activities that were more than 88 and 90% of the original level, respectively. These results indicate that 7H add, like the parental barley cv. Manas, was able to retain its CO_2 fixation rate during salt stress (up to 200 mmol L^{-1} NaCl) with relatively high g_s. Barley is regarded as being more salt tolerant than bread wheat, and the role of the 7H addition in modifying the Na^+ and K^+ contents in the leaves during salt stress has also been reported (Colmer et al., 2006). In connection with this, a previous study reported that the photosynthetic rate was unaffected in a salt-resistant common centaury in this salinity range (Siler et al., 2007). It is thought that the estimation of net assimilation rate could be a good tool for discriminating between salt-tolerant and non-tolerant cultivars (Belkhodja et al., 1999). In light-saturated C_3 plants under normal conditions, A does not reach the maximum level which would otherwise be measurable at saturating CO_2 concentration (A_{max}). As mentioned above, at an intermediate level of salt stress, stomatal closure limits CO_2 fixation (Bongi and Loreto, 1989; Centritto et al., 2003). Enhanced CO_2 concentration, however, led to the recovery of A_{max} (Cornic, 2000; Cornic and Fresneau, 2002). Other studies, however, have reported that a decrease in A may also result from non-stomatal factors (Bongi and Loreto, 1989; Brugnoli and Björkman, 1992; Centritto et al., 2003), such as reduced mesophyll conductance and/or important metabolic factors during osmotic stress (Tezara et al., 1999; Delfine et al., 2001; Flexas et al., 2002; Lawlor and Cornic, 2002; Loreto et al., 2003; Centritto et al., 2003; Chaves et al., 2003). As can be seen in Figure 2, the extent of L_s was similar in all the examined genotypes at mild salt stress (200 mmol L^{-1} NaCl), while L_m increased even at this moderate level in wheat cultivars, indicating the increased role of non-stomatal limitation in the inhibition of A. At the same time, the relative importance of L_m was not significant at this level for Manas or 7H add. In one respect, these results suggest that the processes underlying non-stomatal limitation are important in the case of wheat cultivars but have little significance for 7H add or barley at a moderate stress level. On the other hand, it is well known that osmotic stress (without the direct role of sodium ions) is linked to salt stress (Hsiao, 1986; Joset et al., 1996; Munns, 2002). However, there was only

a slight decrease in RWC in the wheat genotypes at 200 mmol L^{-1} NaCl, suggesting that the higher non-stomatal limitation in wheat lines is not a consequence of water deficit.

When salt stress was severe (300 mmol L^{-1} NaCl) A was strongly inhibited in all the lines, with increased non-stomatal limitation. Nevertheless, L_m was significantly lower for Akom and 7H add than for Manas and Mv9 kr1. It is interesting that the changes in non-stomatal limitation in 7H add were similar to those recorded for Manas at moderate salinity level (200 mmol L^{-1} NaCl, where L_m was low), but resembled those in Akom (where L_m was lower than for Manas) in the case of severe salt stress (300 mmol L^{-1} NaCl).

In conclusion, the results seem to suggest that the newly-developed 7H Akom-Manas addition line is able to maintain a satisfactory level of photosynthetic activity with only minor water loss under moderate salt stress. The better tolerance of the photosynthetic parameters in the parental barley cv. Manas to moderate salinity thus appears to be manifested in this plant. However, these preliminary results require further in-depth analysis in terms of physiology and production biology.

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DETERMINATION OF INCOMPATIBILITY *(S)* GENOTYPES OF SWEET CHERRIES IN THE HUNGARIAN GENE-BANK BY A PCR-BASED METHOD

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The sweet cherry (*Prunus avium* L.) gene-bank collection in Hungary comprises mainly local cultivars. The incompatibility (*S*) genotypes of 48 accessions from the central region of Hungary were investigated by PCR amplification of the intron regions of the *S-RNase* and *SFB* genes responsible for compatibility relationships in sweet cherry. The *S*-genotypes of 38 accessions were completely determined; they showed various pairs of nine alleles and could be assigned to 15 of the existing incompatibility groups or, in the case of three accessions having the novel genotype S_6S_{13}, to the new incompatibility group XLII. For 10 accessions only one *S*-allele could be identified, as a single *S*-RNase product was generated and the intron region of the *SFB* gene of the second allele could not be amplified.

Key words: sweet cherry, *Prunus avium*, genetic resources, incompatibility, *S* allele

Introduction

The fruits of sweet cherry were popular among the early inhabitants of the Carpathian Basin and it is likely that wild cherry may have played a role in the formation of the original local landraces. The Turkish invasion (1541–1686) doubtless introduced west Asian material. Later, cultivars may have been introduced from the Balkans and south Russia. With the arrival of German settlers, western European cultivars appeared and became popular in the 19th century. This diverse genetic material formed the basis for the Hungarian sweet cherry gene-bank established in the 1970s. The development of cherry in Hungary was reviewed by Rapaics (1940).

Sweet cherry (*Prunus avium* L.) is primarily a self-incompatible species. Although self-compatible cultivars are now available, the most widely grown cultivars in Hungary are self-incompatible. Thus, for adequate fruit set, appropriate cross-compatible cultivars are essential in orchards.

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Incompatibility in the Rosaceae family to which the stone-fruit and pome-fruit species belong is gametophytic and controlled by a multi-allelic locus, called the *S*-locus. This comprises a minimum of two genes; one encodes a ribonuclease (*S*-RNase) expressed in the style, whereas the other encodes an F-box protein (*SFB*) expressed in the pollen. Cultivars bearing the same two alleles are cross-incompatible and form an incompatibility group. Molecular methods are now available for determining the *S*-genotype of sweet cherry cultivars and accessions, so cross-compatible cultivar combinations can be predicted rapidly in the laboratory instead of by time-consuming test crosses in the field.

The *S*-genotypes of many commercial sweet cherry cultivars have been collated (Tobutt et al., 2005), among them, various cultivars important to Hungarian growers (Békefi et al., 2003). Recently, the identification of the *S* alleles of German (Schuster et al., 2007), Sicilian (Marchese et al., 2007) and Spanish (Gisbert et al., 2008) gene-bank accessions has been reported. In addition, De Cuyper et al. (2005) and Vaughan et al. (2006) determined the *S*-genotype of the members of wild cherry populations and Papp et al. (2009) focused on the relationship between Hungarian cultivated and wild cherry accessions.

At present, 26 *S*-alleles (S_1 – S_{32}, with duplicates and unfilled numbers) have so far been described in *Prunus avium* (wild and cultivated), and there are currently 41 incompatibility groups among cultivated cherry genotypes.

This study characterises the *S* allele composition of Hungarian sweet cherry gene-bank accessions by PCR analysis. It uses a multiplex PCR method developed by Vaughan et al. (2006), which uses fluorescently labelled primers amplifying across intron regions of the *S-RNase* and *SFB* genes and allows rapid and precise screening of large populations.

Materials and methods

Plant material

The 48 accessions analysed are in the gene-bank maintained by the Research Institute for Fruit Growing and Ornamentals in Érd (Table 1). They are local cultivars of the Central Hungarian region. Cultivars with known *S*-genotypes were included as standards and came from the collection of East Malling Research (Table 2). DNA was extracted from dormant buds according to a CTAB miniprep method (Doyle and Doyle, 1987) as modified by Sonneveld et al. (2001).

Molecular analysis

Genomic DNA was screened using two pairs of fluorescently labelled primers amplifying across the first intron of the *S-RNase* gene, PaSPcons-F1 and PaC1cons-R1 (Sonneveld et al., 2006) and across the intron present in the 5' untranslated region of the *SFB* gene, F-BOX5'A and F-BOXintronR (Vaughan et al., 2006). PCR reactions were performed in a total reaction volume of 8 µl comprising 1.25 ng of plant DNA, 1×Qiagen Multiple PCR master-mix buffer (Qiagen) and 2 µM of each primer. The cycling conditions of the PCR reactions were in accordance with the protocol described by Vaughan et al. (2006). The sizes of the amplification products were determined on an ABI Prism 3100 semi-automated sequencer.

Genotypes were deduced by comparing the scores of the accessions with those of the standard cultivars and intron sizes described by Vaughan et al. (2006).

Table 1
Cherry accessions analysed from the Hungarian gene-bank, their *S*-genotypes and their assignment to incompatibility groups

Accession	*S*-genotype	Incompatibility group
Késői Vadcseresznye	S_1S_3	II
Szeptember	S_1S_3	II
Augusztus Elején Érő A	S_3S_4	III
Csákvári Korai	S_3S_4	III
Disznódi Fűszeres	S_3S_4	III
János Cseresznye	S_3S_4	III
Késői Fekete Nagy	S_3S_4	III
Késői Helyi Fekete A	S_3S_4	III
Szőlősi Pollenadó	S_3S_4	III
Tarka Cseresznye	S_3S_4	III
Újszászi Cseresznye	S_2S_3	IV
Májusi Korai Cseresznye	S_4S_5	V
Fehér Cseresznye	S_3S_6	VI
Fehér Ropogós Pollenadó	S_3S_6	VI
Fehér Cseresznye VK	S_3S_6	VI
Kecskecsöcsű	S_3S_6	VI
Pákozdi Fehér	S_3S_6	VI
Tápiósági Cseresznye	S_3S_6	VI
Augusztusi Fehér	S_3S_5	VII
Cserkeszőlő 7	S_3S_5	VII
Dunabogdányi Szív Alakú Cseresznye	S_1S_4	IX
Késői Tarka	S_1S_4	IX
Cserkeszőlő 2	S_6S_9	X
Dányi Cseresznye	S_6S_9	X
Korai Ropogós A	S_3S_9	XVI
Torbágyi Késői Fekete	S_4S_6	XVII
Péceli	S_3S_{13}	XIX
Augusztus Elején Érő B	S_1S_6	XX
Pomázi Középérésű	S_1S_6	XX
Farmosi Cseresznye	S_3S_{12}	XXII
Korai Ropogós B	S_3S_{12}	XXII
Móri K Cseresznye	S_3S_{12}	XXII
Ropogós Cseresznye A	S_3S_{12}	XXII
Ropogós Cseresznye B	S_4S_{12}	XXVII
Fekete Helyi Cseresznye	S_5S_9	XXXVII
Cserkeszőlő 4	S_6S_{13}	new group (XLII)
Fertődi Csüngő	S_6S_{13}	new group (XLII)
Tasziló	S_6S_{13}	new group (XLII)
Cserkeszőlő 5	$S_3S_?$	
Fertődi Borostyán	$S_3S_?$	
Késői Helyi Fekete B	$S_3S_?$	
Pomázi Hosszúszárú	$S_3S_?$	
Bicskei Fekete	$S_4S_?$	
Kókai Cseresznye	$S_4S_?$	
Szomolyai Legkorábbi	$S_4S_?$	
Prágai Cseresznye	$S_5S_?$	
Gyöngyösi Szívcseresznye	$S_6S_?$	
Vadcseresznye Fekete	$S_{16}S_?$	

Table 2
Cherry cultivars from the East Malling gene-bank used as standards, and their *S*-genotypes

Cultivar	*S*-genotype
Early Rivers	S_1S_2
Napoleon	S_3S_4
Colney	S_5S_6
Orleans 171	S_7S_{11}
Inge	S_4S_9
Schneiders Späte Knorpelkirsche	S_3S_{12}
Noble	S_6S_{13}
Dikkeloen	S_5S_{14}
Strawberry Heart	S_3S_{16}

Results

The genotypes of the accessions analysed were deduced by examining the patterns of product sizes and comparing them with those of standard cultivars of known genotypes (Table 2). PCR product sizes generated for standard cultivars corresponded rigorously to the known *S*-alleles, except for S_2, where the amplification product of the *SFB* gene was missing in standard cultivar Early Rivers. However, the *S*-RNase and *SFB* intron sizes of Újszászi Cseresznye obviously indicated S_2. The *S*-haplotype of 38 local cultivars among the 48 accessions studied was fully determined (Table 1). Nine different *S* alleles (S_1, S_2, S_3, S_4, S_5, S_6, S_9, S_{12} and S_{13}) were detected in 16 combinations, representing 15 existing incompatibility groups, and in the case of three accessions scored as S_6S_{13}, a new incompatibility group was designated, XLII. The remaining 10 accessions could only be partially genotyped, as only one *S*-allele could be rigorously confirmed due to non-amplification of the *SFB* intron. The size of the ribonuclease product of the problematic allele was 346 or 347 in all cases. This allele was often connected with S_3 or S_4 alleles. Examples of electrophorograms generated for genotypes S_3S_{12} and $S_4S_?$ are presented in Figure 1.

Among the local cultivars analysed, the most common allele was S_3, which was present in 66% of the accessions. S_{13}, which is typically very rare in sweet cherry, was also detected in four accessions. The relative allele frequencies observed were compared with the occurrence of these alleles in international cherry cultivars, reported by Bošković and Tobutt (2001) (Fig. 2).

Discussion

In this work, the *S*-allele constitution of 48 sweet cherry accessions (local cultivars originating from the Central Hungarian region and kept in the Hungarian gene-bank) was characterised. In the case of 38 accessions, both *S*-alleles could be identified and corresponded to the alleles described in sweet cherry.

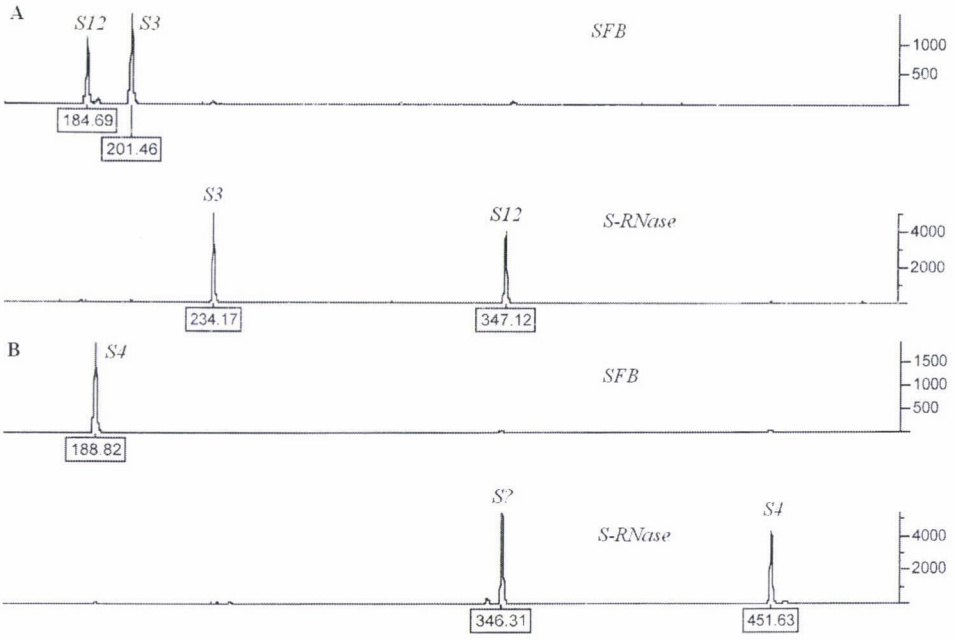

Fig. 1. Electrophorograms displaying traces for *S-RNase* first intron and *SFB* intron products generated for genotypes S_3S_{12} (A) and $S_4S_?$ (B)

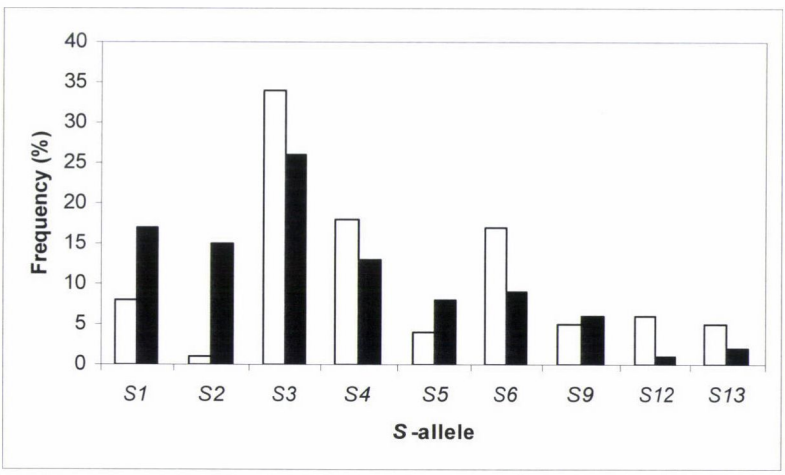

Fig. 2. Relative occurrence of the *S*-alleles detected in 38 Hungarian cherries (white column) compared with the frequencies found in 67 international cultivars by Bošković and Tobutt (2001) (black column)

The genotype of Cserkeszőlő 4, Fertődi Csüngő and Tasziló appeared to be S_6S_{13}. Such a genotype has not been reported previously; thus a new incompatibility group (XLII) is proposed.

Some accessions with identical names were found to differ in their *S*-genotypes. One example is Augusztus Elején Érő, where one accession is S_3S_4, whereas the other is S_1S_6. Thus, these accessions cannot be regarded as identical and have been labelled differently, by adding discriminating letters (A and B) after the accession names. Other examples are Késői Helyi Fekete, Korai Ropogós and Ropogós Cseresznye. This indicates that having genetic profiles for the accessions allows much more thorough archiving and management of gene-bank resources.

A comparison of allele frequencies showed that the most frequent allele was S_3, as also reported by Bošković and Tobutt (2001), who analysed mainly international cultivars grown in Western Europe, and by Békefi et al. (2003), who analysed cultivars important in Hungary. The present study found the S_4 allele in many accessions; this allele was frequent in the cultivars analysed by Bošković and Tobutt (2001), but was uncommon or absent in the case of Sicilian (Marchese et al., 2007) and Spanish (Gisbert et al., 2008) local cultivars. Similar to the findings of these latter authors, the S_1 and S_2 alleles were found to be less frequent as compared with international cultivars; in contrast, S_6 was relatively frequent. The allele S_{13}, which appeared in many wild cherry accessions (De Cuyper et al., 2005; Vaughan, unpublished data) and in Sicilian cultivars (Marchese et al., 2007), was more frequent in the Hungarian accessions than in international cultivars. These results show similarities between South European and Central European cultivars. The S_{14} allele, along with the S_{17}-S_{22} alleles that often appear in wild cherries, were missing from the Hungarian accessions, indicating the result of human selection among sweet cherry types grown for their fruit.

Knowledge of the *S*-genotypes of different accessions further enables the determination of relationships within cultivated fruit species, as has been achieved in apricot (Halász, 2007). Therefore, *S*-allele analysis is recommended for the local sweet cherry cultivars of other regions of Hungary, as well as for wild cherry, which is native to Hungary.

It proved impossible to detect the intron region of the *SFB* gene for 11 accessions. The size of their *S*-RNase first intron PCR amplification products ranged from 346.2–346.7 bp, which could be indicative of the S_2, S_7 or S_{12} alleles. The *SFB* intron products of these alleles should be 187, 181 and 185 bp, respectively, but they did not amplify. Vaughan et al. (2006) observed problems in amplifying the *SFB* for S_9 and S_{14}, which they attributed to the deviation of the primer sites or differences in the surrounding DNA conformation of these loci. However, these authors indicated that the S_2, S_7 and S_{12} alleles can be easily distinguished using *SFB* primers. The *S-RNase* patterns of the 10 accessions were not suggestive of the S_9 or S_{14} alleles. These accessions should be analysed using allele-specific primers to see whether they have S_2, S_7 or S_{12} alleles or a new allele. The failure to amplify the *SFB* intron for S_2 in the case of the standard cultivar Early Rivers may indicate that the unidentified allele is S_2.

Recently, farmers have turned towards traditional local cultivars and it is thought there may be a role for these in chemical-free ecological production. For such orchards, knowledge of incompatibility relationships between the cultivars is essential. Several accessions studied are cross-incompatible with widely grown Hungarian cultivars. For instance, Farmosi Cseresznye, Korai Ropogós B, Móri K and Ropogós Cseresznye A (Group XXII) are incompatible with the popular cultivars Germersdorfi 1, Germersdorfi 3 and Linda, belonging to the same group (Békefi et al., 2003). Sweet cherry cultivars with dark juice colour are popular among growers as they are suitable for deep freezing. Gyöngyösi Szívcseresznye, Fekete Helyi Cseresznye and Móri K, all of which have dark juice colour, are compatible with each other and with Szomolyai Fekete, the main cultivar used for deep freezing in Hungary, genotyped by Békefi et al. (2003); so these accessions may be grown together in orchards. The accession Szomolyai Legkorábbi shares a common allele, S_4, with Szomolyai Fekete, previously genotyped as S_2S_4 (Békefi et al., 2003); if this is essentially a clone of the same cultivar the unidentified allele of the former is presumably S_2.

Fruit breeders always try to satisfy consumer demands and gene-bank collections serve as a basis for their work. The results described here will enable breeders to make better use of gene-bank resources to choose cross-compatible combinations for sweet cherry.

It is recommended that *S* genotyping should be carried out for local sweet cherry cultivars of other regions of Hungary, as well as for wild cherries native to Hungary.

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MANGANESE AND ZINC CONCENTRATIONS IN MAIZE GENOTYPES GROWN ON SOILS DIFFERING IN ACIDITY

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Drought and soil acidity are two major abiotic stress factors limiting maize production worldwide, generating imbalances in the manganese (Mn) and zinc (Zn) status in plants. This study was conducted to determine the effects of drought stress on the Mn and Zn status in maize genotypes grown on acid and non-acid soils and how the Mn and Zn status affects the changes in grain yield caused by drought stress and soil acidity. Seventeen genotypes were grown at two locations differing in soil acidity in Eastern Croatia in 2003 and 2004. Positive values of an aridity index indicated drought stress in 2003. The genotypes had much higher Mn and Zn concentrations on acid soil than on non-acid soil: more than twice as high in both seasons for Zn and about 6 and 9 times higher in normal and in dry seasons, respectively, for Mn. This demonstrates that drought combined with soil acidity led to the excessive accumulation of Mn in maize plants. However, variation was observed between the maize genotypes for the Mn accumulation on soils differing in acidity when drought occurred. Some genotypes accumulated Mn on acid soil irrespective of drought. The Mn and Zn status had no discernible effect on the changes in grain yield caused by drought stress and/or soil acidity.

Key words: drought stress, genotypic variation, grain yield, maize, manganese, soil acidity, zinc

Introduction

Certain levels of micronutrients are necessary to mediate the numerous biochemical reactions essential for the growth and development of crop plants. The importance of several micronutrients in maize (*Zea mays* L.) is well documented (e.g. Mengel and Kirkby, 2001), emphasizing the vulnerability of maize plants to nutrient deficiencies, excesses and imbalances. Since maize is a zinc-demanding plant, special attention should be given to possible problems with the zinc (Zn) balance in soil and plant. However, other nutritional problems may also arise in maize production, for example either a deficiency or excess of manganese (Mn). Zn and Mn uptake is closely related to the status of other

nutrients in plant and soil, causing changes in the most important trait in maize, the grain yield, and emphasising the necessity of studying interrelationships between nutrient contents in the soil–plant system.

Kovacevic et al. (2004) found that heredity seems to have more influence on the nutritional status of maize than environmental factors, based on the considerably greater genotypic differences observed when the available Zn and Mn in the soil were adequate for plant needs. This situation changed, however, if the maize was exposed to abiotic stress factors. Acid soils (pH_{water}<5.6) occupy millions of hectares worldwide (von Uexkull and Mutert, 1995), adversely affecting maize yields by increasing the solubility of Al and Mn to toxic levels and by reducing the availability of phosphorus (P) (Sumner and Farina, 1986). Kovacevic et al. (1997) tested the Zn and Mn nutritional status of the ear-leaf in four genetically diverse inbred lines of maize as parents and their 12 single-cross hybrids on acid soil. Generally, considerable differences in Zn and Mn status were found between the parents under the same environmental conditions and this was reflected in their progeny. However, no comparison was made between the nutrient concentrations on acid and non-acid soil.

Drought, responsible for the major limitation of crop yields worldwide (Boyer, 1982), is another important abiotic stress factor in maize production. Mn leaf accumulation has been shown to be related to certain aspects of drought tolerance in soybean (Purcell et al., 2000; Vadez and Sinclair, 2002; Sinclair et al., 2007) and cotton (Weil et al., 1997). Very few results have been published on the relationship between Mn status and drought tolerance in maize genotypes (De et al., 2004).

It is therefore important to study the genotypic variation in the drought stress tolerance of maize genotypes under varied Zn and Mn conditions in the soil–plant system. This study was conducted to determine the effects of drought stress on Zn and Mn status in 17 maize genotypes grown on acid and non-acid soils. It was also aimed to study how the Mn and Zn nutritional status of the plants affects the changes in grain yield caused by drought stress and soil acidity.

Materials and methods

Seventeen maize genotypes were grown under field conditions in three replications (randomized complete block design) in Eastern Croatia in the 2003 and 2004 growing seasons. The two fields are located in a region suitable for growing FAO 400 to 500 maize genotypes at 45°N (latitude) and 18°E (longitude) with an approximate elevation of 200 m above sea level (localities Gundinci – non-acid soil and Zelcin – acid soil) with markedly different soil chemical properties (Table 1). The pH_{water} of the acid soil was sufficiently low to promote the excessive availability of Mn, but high enough that Al toxicity was unlikely to be a problem. The soil types were determined according to the WRB classification (FAO/ISRIC/ISSS, 1998) as eutric gleysol (non-acid soil) with 3.1% sand, 62.8% silt and 34.1% clay (silty clay loam) and stagnic luvisol (acid soil) with 13.2% sand, 74% silt and 12.8% clay (silty loam). A comparison of the air capacity showed 9.18% vol. for the acid soil and only 2.11% vol. for the non-acid soil, suggesting soil compaction in the eutric gleysol. The usual soil and crop management practices for maize were applied in both locations and years. The relative proximity of the sites (around 50 km apart) should minimize climatic differences, emphasizing the genotypic and edaphic effects within a particular growing season.

Table 1
Chemical properties of two soils (0–30 cm) differing in acidity in two maize growing seasons

Soil/Year	pH		Organic matter	mg kg^{-1} of soil		
	water	1 M KCl	(%)	P_2O_5	Mn	Zn
Non-acid 2003	6.8	5.8	2.4	245	22.4	0.9
Non-acid 2004	7.2	6.6	2.9	229	12.6	1.2
Acid 2003	5.2	3.9	1.5	75	65.7	1.6
Acid 2004	4.8	3.9	2.0	96	56.8	2.5

An aridity index (AI) was estimated in order to quantify the combination of dry and warm weather during June, July and August, the critical times for stress that could eventually have an impact on grain yield. The index of aridity for each month (i) and year (j) is given by $AI_{ij} = T' - Pr'$, where T' and Pr' are standardized monthly temperature and precipitation, respectively, calculated as $T' = (T - Tbar) / St$, and $Pr' = (Pr - Prbar) / Spr$, where T is the monthly temperature, Tbar the average monthly temperature over all years, and St the standard deviation of the monthly temperature over all years. Likewise, Pr is the monthly precipitation, Prbar the monthly mean precipitation over all years, and Spr the standard deviation of the precipitation over all years. Finally, three AI_{ij} values were summed to give AI_j, the seasonal aridity index, or simply AI. Positive and high values of AI indicate a warmer and drier season than normal. This index is a modification of the AI proposed by Harouna and Carlson (1994).

Ear-leaf samples were taken at the beginning of the silking stage for chemical analysis (approximately 25 leaves in the mean sample) from each plot. Mean soil samples were taken using an auger at a depth of 30 cm. The concentrations of P, Mn and Zn in the maize leaves were determined using the inductively coupled plasma (ICP) technique after microwave digestion. The leaf samples were digested in 65% nitric acid (HNO_3) + 30% hydrogen peroxide (H_2O_2) using a Milestone MLS 1200 microwave. Analyses were performed with a Jobin-Yvon Ultrace 238 ICP-OES spectrometer. The mobile fraction of these elements in the soil was also determined by ICP after extraction with ammonium acetate-EDTA (pH 4.65) solution using the Lakanen and Erviö (1971) method. Plant and soil analysis were conducted in the laboratory of the Research Institute for Soil Science and Agricultural Chemistry (RISSAC), Budapest, Hungary. All compositional data were calculated on a dry matter basis. Grain yields were calculated on a 14% grain moisture basis.

Initially, data from each environment were statistically analysed separately. Entry means and error mean squares were used for further combined analyses of variance (Cochran and Cox, 1957). Outliers were detected according to Anscombe and Tukey (1963) and if significant at P= 0.05, they were declared to be missing values and estimated using the iterative method of Healy and Westmacott (1956). There were less than 1% of outliers in the whole data set. The data were then subjected to combined analyses of variance, firstly using a two-factor model including genotypes and four environments as main factors, and secondly with a three-factor model that included soil type (acidity), year and genotype as main factors. Genotype was considered as a fixed effect in all the analyses, while the four environments were considered as random in the two-factor model, and soil type (acidity) and year as fixed effects in the three-factor model. The PLABSTAT program package (Utz, 1995) was used for all the statistical procedures in the study.

Results

There were notable differences in precipitation and air temperatures between the two growing seasons (Table 2). During the 2003 growing season, lower precipitation and higher temperatures were recorded than during the 2004 growing season. The highly positive values of AI in 2003 demonstrate a much drier and warmer season than normal, indicating drought stress, while the AI value in 2004 showed that the growing conditions were not dry.

Table 2

Precipitation (mm), air temperatures (°C), and seasonal aridity index (AI) in Eastern Croatia (Osijek) for three months during two maize growing seasons. (Sources: Meteorological and Hydrological Bulletin Service of the Republic of Croatia, 2003, 2004)

Year	June		July		August		AI
	mm	°C	mm	°C	mm	°C	
2003	44	24.3	61	22.1	41	23.6	7.2
2004	88	19.8	58	22.1	105	21.4	-0.2
Average*	83	19.8	66	21.2	58	20.8	0.7

*Average for 20-year period 1984–2004

The mean values for all five traits across four environments differed significantly (Table 3), and this was associated with specific abiotic stress, either drought or soil acidity. On average, the maize genotypes had the lowest grain yield under dry conditions. While the grain moisture varied significantly according to the drought stress pattern, the values of P, Mn and Zn concentrations in the maize leaves varied according to soil acidity. Although significant, the P concentrations were not markedly higher on non-acid soil. In contrast, the maize genotypes had much higher average Mn and Zn concentrations on acid soil: more than twice as high for Zn in both seasons and about 6 or 9 times higher in normal and dry seasons, respectively, for Mn.

Three-factor analysis of variance revealed highly significant effects of soil type (S) and genotype (G) for all five traits investigated, while the effect of year (Y) was highly significant only for grain yield, grain moisture and Mn concentration (Table 4). There was no significant year effect for the P and Zn concentrations. Both the twofold interactions of soil type with year (SY) and genotype (SG) were only highly significant for Mn concentration. The 3-way interaction SYG was only highly significant for grain yield and Zn concentration.

Table 3

Average grain yield (t ha^{-1}), grain moisture (%) and concentrations of P, Mn, and Zn in ear-leaves of 17 maize genotypes grown in four environments differing in soil acidity and drought stress, with least significant differences (LSD) at the 0.05 probability level

Environment	Grain yield	Grain moisture	P (mg g^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
Non-acid/Drought	3.87	23.86	0.36	24.63	25.09
Non-acid/No drought	6.61	25.55	0.38	26.64	23.13
Acid/Drought	5.68	24.68	0.30	216.16	50.52
Acid/No drought	7.09	30.64	0.30	152.36	52.33
LSD (0.05)	0.60	1.18	0.02	17.12	3.84

Table 4

F-statistics and significance levels of the mean squares for soil type (S), year (Y), genotype (G), respective two-fold interactions (SY, SG, YG), and the three-fold interaction (SYG) for two agronomic and three quality traits (P, Mn and Zn concentrations in ear-leaves) combined across four environments

Source	Grain yield	Grain moisture	P	Mn	Zn
S	26.38**	158.29**	115.92**	1295.07**	629.64**
Y	86.36**	264.41**	2.12ns	49.12**	0.00ns
G	27.47**	13.42**	10.19**	4.65**	4.11**
SY	8.84**	82.66**	0.8ns	55.73**	2.98ns
SG	0.55ns	1.95+	2.13+	3.67**	3.08*
YG	1.15ns	6.41**	1.04ns	0.93ns	0.54ns
SYG	3.18**	0.87ns	1.33ns	2.00*	2.51**

+, *, ** Significant at the 0.1, 0.05 and 0.01 probability levels, respectively; ns: non-significant.

The average Mn concentrations of the maize genotypes differed significantly within a particular environment (Fig. 1). Although less visible, there was much greater variation between the genotypes in a non-acid/dry environment compared to the non-acid/non-dry environment: the Mn concentrations ranged from 9.2 to 49.2 mg kg^{-1} in the dry season and from 15.7 to 38.1 mg kg^{-1} in the normal season. Genotype 1 had consistently the highest and genotype 15 consistently the lowest values of Mn concentration on non-acid soil. Much wider differences between the genotypes occurred on acid soil in both seasons, with excessive Mn accumulation of 132.7 to 296.0 mg kg^{-1} in the dry season and 108.0 to 241.3 mg kg^{-1} in the normal season. Genotype 14 consistently had the highest Mn concentrations, while the lowest Mn values were recorded for genotype 16. Interestingly, genotype 15 also had one of the lowest concentrations in both seasons on acid soil.

The associations between grain yield and compositional traits varied across the four environments (Table 5), but the tightest associations were found for the non-acid/dry environment, where the relationship between grain yield and Zn concentration was significantly negative at the 0.05 probability level. There was a moderate positive correlation between grain yield and Mn concentration on non-acid soil, but this was only weak or very weak on acid soil. There was no correlation between the P and Mn concentrations on non-acid soil, but this relationship was positive, significant and strong on acid soil in both seasons. There was a weak negative Mn–Zn association on non-acid soil, and a highly significant positive association on acid soil.

Discussion

Interest in the variation between crop genotypes for tolerance of abiotic stress has generated a huge amount of literature. In maize, genetic variability was studied mostly for drought (e.g. Bänzinger et al., 2006) and soil acidity (e.g. Baligar et al., 1997), but also for heat (Karim et al., 2000) and soil compaction

(Soyelu et al., 2001). However, most of these studies were conducted under fully controlled conditions in a greenhouse. When maize experiments are conducted in the field, all investigations should include complex genotype × environment interactions, which are a common feature of research on abiotic plant stress. The issue of genotype × environment interactions is even more complex when several types of abiotic stress occur. For example, when adverse physical soil properties interact with environmental variation in the timing and severity of water deficits, with genetic variation in flowering time, or with nutrient deficiencies and toxicities, whose occurrence and severity interact with water deficits (Bänziger and Cooper, 2001). This could be particularly important in low-income countries where a technological gap exists between soil management and soil properties (Fournier et al., 2007).

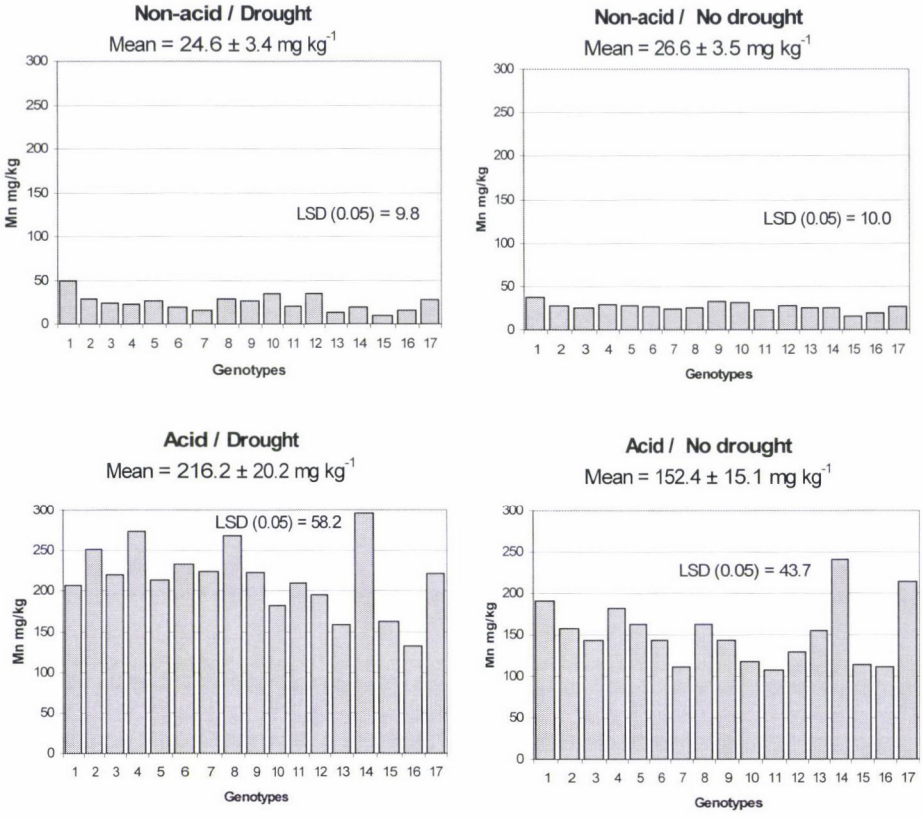

Fig. 1. Average manganese concentrations in ear-leaves, with least significant differences (LSD) at the 0.05 probability level, for 17 maize genotypes grown in four environments differing in soil acidity and precipitation

Table 5

Coefficients of correlation between two agronomic traits and the ear-leaf concentrations of phosphorus (P), manganese (Mn) and zinc (Zn), investigated in 17 maize genotypes in four environments differing in soil acidity and precipitation

Environment Trait	Grain yield	Grain moisture	P	Mn
Non-acid/Drought				
Grain moisture	0.32			
P	–0.52*	–0.04		
Mn	0.50*	0.07	0.07	
Zn	–0.74**	–0.09	0.61**	–0.33
Non-acid/No drought				
Grain moisture	–0.10			
P	–0.47	0.25		
Mn	0.47	–0.12	0.09	
Zn	–0.10	0.58*	0.35	–0.18
Acid/Drought				
Grain moisture	0.18			
P	–0.37	0.32		
Mn	0.12	0.44	0.53*	
Zn	0.23	0.31	–0.08	0.64**
Acid/No drought				
Grain moisture	–0.60*			
P	–0.60*	0.52*		
Mn	–0.32	0.12	0.77**	
Zn	–0.10	–0.17	0.55*	0.68**

*, ** Correlation coefficient significant at the 0.05 and 0.01 probability levels, respectively

The results presented in this study demonstrate that water deficit combined with soil acidity led to an extremely high accumulation of Mn in maize plants. According to Christensen (cit. Mengel and Kirkby, 2001), for maize to have normal nutritional status the leaf Mn concentration under standard fertilization conditions should be from 20 to 200 mg kg^{-1}, suggesting that the increase in Mn on acid soil reached toxic levels in some genotypes in the present study. While the Zn concentrations exhibited neither deficiencies nor excesses on either soil type (normal status for Zn is from 20 to 70 mg kg^{-1}), the Mn status on non-acid soil was in some cases under the lower limit (less than 10 mg kg^{-1} Mn). This suggests great flexibility in maize genotypes as regards their Mn accumulation during drought when grown on non-acid or acid soil. Khan and McNeilly (1998) found considerable variability between 72 accessions of maize for Mn leaf concentrations in standard solution culture, where some genotypes exhibited tolerance to Mn toxicity at the seedling stage.

The Mn and Zn nutritional status of the plants had no discernible effect on the changes in grain yield caused by drought stress and/or soil acidity. The inconsistent correlation coefficients between grain yield and Mn and Zn status across the four environments suggest that grain yield as a complex trait was not predetermined by Mn or Zn status, even under stress conditions. However, there

was a positive moderate relationship between Mn status and grain yield on the compacted (non-acid) soil, especially under dry conditions. De et al. (2004) found that Mn fertilizer could improve stomatal conductance, net photosynthetic rate and water use efficiency in maize, which could eventually lead to higher grain yield. Consequently, the increased Mn availability in the acid soil in the present study should contribute to the drought tolerance of maize.

It can be concluded that variation exists between maize genotypes for the accumulation of Mn on soils differing in acidity when drought stress occurred. This may be due to differential tolerance to acid soil and/or the interaction between acid soil tolerance and drought tolerance, as demonstrated in this study by the highly significant twofold interactions of soil type with the precipitation regime and with genotype. Further work is required to clarify these issues. There appears to be no universal mechanism of Mn accumulation in maize genotypes on acid soils with different precipitation regimes. However, some genotypes can accumulate similar quantities of leaf Mn, irrespective of drought sress (e.g. genotype 17), also having similar Mn status on non-acid soil, again irrespective of drought.

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EFFECTS OF DROUGHT STRESS ON BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS IN CALLUS CULTURES OF *Carthamus tinctorius* VARIETIES

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The aim of the work was to evaluate the callus induction response and *in vitro* drought tolerance of eight genotypes of safflower. The experiment was laid out as a completely randomized design in a factorial arrangement with three replications. To evaluate the drought tolerance of the genotypes, growing calli were exposed to drought stress after two subcultures by adding different concentrations of mannitol to the culture medium for one month. Under stress conditions, the genotypes were compared in terms of proline content, cell viability, relative growth rate, ion content (Na^+ and K^+), relative water content and index of tolerance. Drought affected all the measured biochemical and physiological factors and there were significant differences between the tested genotypes. The proline content increased in drought-stressed calli, and mannitol, as a stress agent, stimulated the synthesis of proline in all the genotypes, especially at the highest concentration (505 mM), whereas the ion contents, cell viability, RWC, RGR and index of tolerance exhibited a significant decrease. This suggested that these biochemical and physiological traits could be used to predict the drought tolerance of safflower genotypes. The results indicated that the cultivars Isfahan and LRV-51-51 were more drought-tolerant under *in vitro* conditions than the other genotypes.

Key words: *in vitro* water stress, safflower, callus induction, cell viability, relative growth rate

Abbreviations: 2,4-D: 2,4-Dichlorophenoxyacetic acid; NAA:α-Naphthaleneacetic acid; BAP: 6-Benzylaminopurine; MS: Murashige and Skoog medium; RWC: Relative water content; RGR: Relative growth rate; TTC 2,3,5-Triphenyltetrazolium chloride

Introduction

Safflower (*Carthamus tinctorius* L.), one of the world's oldest oil seed crops with 35–40% oil content, is grown commercially as a source of edible oil and natural dyes throughout the world. Water stress is a major abiotic stress reducing the yield of a wide variety of crops all over the world. As safflower is more drought-tolerant than some other oil seed crops, it is especially suited for

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dry areas where other oil seeds are difficult to grow (Weiss, 2000). Most of the cultivated area in Iran is rainfed, with low rainfall with high spatial and temporal variability. Safflower has been reported to be a suitable crop under rainfed conditions as an autumn- or spring-sown crop with relatively wide adaptability both to climatic variations and to soils (Esendal, 1997).

Drought-induced osmotic stress triggers a wide range of perturbations, ranging from growth and water status disruption to the modification of ion transport and uptake systems (Santos-Diaz and Ochoa-Alejo, 1994a; Bajji et al., 2000). Generally, plants accumulate some kind of organic or inorganic solute in the cytosol to raise osmotic pressure, thereby maintaining both turgor and the driving gradient for water uptake (Rhodes and Samaras, 1994). Among these solutes, the accumulation of proline has been advocated as a parameter of selection for stress tolerance (Yancy et al., 1982; Jaleel et al., 2007). It has been suggested that an increase in free proline levels is a symptom of injury that results from imbalances in other pathways (Bhaskaran et al., 1985; Perez-Alfocea and Lahrer, 1995). Besides organic solutes, Na^+ and K^+ are also thought to be involved in osmotic adjustment in both the vacuoles and cytosol of numerous plant species (Pitman, 1981; Martinez et al., 2003; 2004). *In vitro* viability tests are considered a credible tool for the characterization and quantification of cell death in plant tissue exposed to stress conditions (Porter et al., 1994). In cell cultures it is very important to have an effective and cheap method to determine clearly and efficiently the presence of viable and non-viable cells. It has been established that 2,3,5-triphenyltetrazolium chloride (TTC), a water-soluble, colourless compound, can be reduced to water-insoluble red formazan by a variety of organisms. TTC reduction is used as a quantitative method in the evaluation of tissue viability. In addition, TTC reduction has been widely used in the viability assay of plant tissues exposed to stressful conditions (Steponkus and Lanphear, 1967). Another parameter, relative water content (RWC), is related to drought resistance and has also been proposed as a more important indicator of water status than other water potential parameters under drought stress (Dhanda and Sethi, 1998; Keles and Oncel, 2004).

Upon exposure to water deficit, plants exhibit physiological, biochemical and molecular responses at both the cellular and whole plant levels (Greenway and Munns, 1980; Hasegawa et al., 2000; Ghasempour and Kianian, 2007). However, the study of these processes at whole plant level is rather difficult. Whole plants contain mostly non-growing cells, which makes it difficult to characterize the biochemical processes that are operating in growing cells in response to abiotic stresses (Turner and Jones, 1980; Neumann, 1997). *In vitro* culture systems are useful for the evaluation of tolerance to environmental stresses because the stress conditions can be easily controlled. Moreover, *in vitro* culture provides a uniform population of synchronously developing plant cells without involving regulatory mechanisms that naturally repair damage at the whole plant level (Tal, 1983). Although various *in vitro* studies focused on

various aspects of plant regeneration in safflower (Nikam and Shitol, 1999; Radhika et al., 2006; Başalma et al., 2008), little is known about the physiological parameters affecting safflower *in vitro* cultures. Reports about the response of safflower callus to drought stress are lacking. Therefore, changes in *in vitro* drought tolerance factors were evaluated in order to screen for drought-tolerant genotypes. For this purpose, the calli of eight cultivars were exposed to varying degrees of mannitol-induced drought stress to determine the physiological and biochemical responses to drought stress in *Carthamus tinctorius*.

Materials and methods

Plant materials

Eight genotypes of *Carthamus tinctorius* L. (LRV-51-51, Lesaf, Cyrian, Gila, Kino-76, Isfahan, Hartman and Cyprus Bregon, labelled G1, G2, G3, G4, G5, G6, G7 and G8, respectively) were provided from the experimental farm of the Faculty of Agriculture, Razi University, Iran in late September 2007.

Seed sterilization

Seeds of *C. tinctorius* L. were surface sterilized with 0.1% mercuric chloride for 7 min, followed by rinsing three times for 5 min in sterilized distilled water. The seeds were then germinated and grown on MS medium (Murashige and Skoog, 1962) with 2% sucrose and 7% agar. The pH of the medium was adjusted to 5.8 with 0.1 N HCl before autoclaving for 20 min at 121°C.

Callus induction

Hypocotyls were excised from 5–7-day-old seedlings and divided into segments of 0.5–1.0 cm. The explants were inoculated on MS medium supplemented with sucrose (2%), agar (0.7%) and growth regulators: α-naphthaleneacetic acid (0.5 mg l^{-1}) in combination with BAP (0.5 mg l^{-1}) and 2,4-dichlorophenoxyacetic acid (0.5 mg l^{-1}) for callus induction. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The induced calli were subcultured three successive times at 30-day intervals in the same medium composition. All the cultures were maintained at 25±1°C under a 16 h photoperiod (PPFD 36 μmolm^{-2} s^{-1}) and 55–60% relative humidity.

In vitro *drought treatment*

After 4 weeks the calli were weighed and transferred to MS medium either without stress or under artificial drought stress conditions using different concentrations of mannitol (180, 350, 505 mM), corresponding to osmotic potentials of –0.78, –1.24 and –1.69 MPa, determined with an osmometer. The calli were maintained in their respective treatments for two subsequent subcultures (one month).

Callus growth

After two subcultures, the growing calli were used for a callus growth study. The calli were weighed before their transfer to fresh callus induction medium (W_0). Then they were weighed again after 4 weeks of culture (W_1). The relative callus growth rate (RGR) was measured according to the following formula:

RGR = [(Final weight – initial weight) × 100]/initial weight (Errabi et al., 2006).

To compare cultivar-related responses to stress conditions, an index of tolerance (INTOL), based on RGR, was calculated according to the following formula:

$$INTOL = RGR_{treatment}/RGR_{control}$$

Determination of relative water content (RWC) in callus

Callus samples of known fresh mass were dried in an oven at 65°C for 48 h, after which they were reweighed and the difference in mass was determined. The water content was expressed as a percentage of callus fresh mass (Al-Khayri and Al-Bahrany, 2004). The callus water content was calculated as (fresh weight – dry weight)/dry weight.

Determination of ion (Na^+ and K^+) content

The Na^+ and K^+ contents of calli treated with or without mannitol were assayed by the flame photometery method. Fresh calli of each treatment were rinsed with distilled water several times and blotted with tissue paper. Then 0.1 g of each callus was powdered and added to 2 ml 3% sulphosalicylic acid solution (SSA). The samples were centrifuged for 10 min at 1000 rpm, then filtered through a Whatman No. 4 filter paper after 48 hours. The filtered solutions were used to determine ions. Each sample solution was diluted with distilled water. The sodium and potassium contents of each sample were then measured with a flame photometer.

Determination of cell viability

Cell viability was determined by the TTC method according to Lutts et al. (2004). Callus tissues (50 mg) treated with different mannitol levels were rinsed in deionized water containing 0.05% Tween-20 and then incubated at 30°C in darkness in tubes containing 5 ml of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) in 50 mM K_2HPO_4 (pH 7.0) for 15 h. The samples were filtered through Whatman No. 4 filter paper, rinsed with deionized water and incubated in 3 ml 94% ethanol at 80°C for 5 min under gentle agitation (80 rpm) to ensure homogenization during the extraction. The samples were then centrifuged at 5000 *g* for 1 min, and the extracted formazan was quantified spectrophotometrically at 487 nm. The viability index was defined according to the absorbance measured per g FW.

Proline measurement

The free proline content in the callus tissues was determined according to Bates et al. (1973). About 500 mg callus tissue was homogenized in 5 ml 3% aqueous sulphosalicylic acid. The filtered homogenate (2 ml) was reacted with 2 ml each of acid ninhydrin and acetic acid and boiled for 1 hour at 100°C. The reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene and mixed vigorously with a stirrer for 10–15 sec. The chromophore containing toluene was aspirated from the aqueous phase and warmed to room temperature. The absorbance was recorded at 520 nm, using toluene as a blank. The proline concentration (μmol 100 mg^{-1} FW) was determined from a standard curve.

Results and discussion

Callus induction and effect of drought stress on callus growth

In a preliminary experiment a medium was designed for callus production in safflower. MS medium supplemented with NAA (0.5 mg l^{-1}), 2,4-D (0.5 mg l^{-1}) and BAP (0.5 mg l^{-1}) proved to be the best medium for callus induction from hypocotyl explants (data not shown). In this medium a suitable amount of calli for transfer to drought stress conditions was obtained after two subcultures. Pieces of calli were placed on a solid selection medium with different concentrations of mannitol and analysed for various physiological traits (for details, see Materials and methods section). Analysis of variance (ANOVA) for all measured traits revealed significant differences between the eight genotypes,

while the genotype × medium interaction also showed a significant difference for all the measured traits except sodium content (Table 1). The results indicated the presence of a considerable amount of genetic variation among the genotypes, especially under drought stress conditions. Reduced growth of tissues has frequently been reported in stressful media (Das et al., 1990; 1992; Misra et al., 1996; 1997a, b) and this has been interpreted as the channeling of the metabolism to resist stress.

In the present study a decrease in RGR and RWC was detected for all the genotypes parallel with increasing mannitol concentration (Tables 2 and 3). When mannitol was added at high concentration (–1.69 MPa) a substantial decrease in the RGR and RWC parameters was observed in calli obtained from all the cultivars (Figs. 1 and 2). Similar trends were observed in the callus of date palm (Al-Khayri and Al-Bahrany, 2004) and in cell clones of chilli pepper (Santos-Diaz et al., 1994b). Callus growth was also expressed as an index of tolerance (INTOL) to eliminate inherent differences associated with the RWC of the eight genotypes in response to stress. Increasing mannitol concentration was associated with a reduction in INTOL for safflower callus (Fig. 3). This result is in agreement with previous research in drought-stressed callus of date palm (Al-Khayri and Al-Bahrany, 2004).

Table 1

Analysis of variance (ANOVA) for physiological traits of calli of eight genotypes subjected to drought stress generated by mannitol

S.O.V.	df	Mean squares						
		Proline	Sodium	Potassium	RGR	RWC	INTOL	Cell viab.
Medium (M)	3	10223.03**	49.09**	3187.6**	4.82**	905.88**	8.36**	105.81**
Genotype (G)	7	3071.1**	33.07**	214.8**	0.35**	48.61**	0.16**	50.88**
G × M	21	296.3**	4.63^{ns}	41.47**	0.16**	19.78**	0.05**	2.68**
Error	64	22.58	3.68	3.43	0.008	2.61	0.008	0.303
CV%		9.58	29.67	5.31	28.11	12.44	29.11	7.6

ns = non-significant; *, **= significant at the 0.05 and 0.01 probability levels, respectively; S.O.V. = source of variance; INTOL = index of tolerance; RGR = relative growth rate; RWC = relative water content

Table 2

Mean comparison of physiological traits in four media

	Cell viability (g^{-1} FW)	INTOL	RWC %	RGR (g g^{-1} FW)	$Potassium^+$	$Sodium^+$	$Proline^+$
M1	9.274^a	1.01^a	21.33^a	0.77^a	49.55^a	7.945^a	28.24^d
M2	8.526^b	0.14^b	13.69^b	0.15^b	37.41^b	6.287^b	38.40^c
M3	6.596^c	-1.041^c	9.641^c	-0.08^c	30.50^c	5.378^{bc}	56.83^b
M4	4.590^d	-0.360^d	7.341^d	-0.24^d	22.32^d	4.622^c	74.98^a

$^+$: µmol g^{-1} d.wt.; Means followed by the same letter(s) in each column are not significantly different (Duncan's Multiple Range Test 5%). INTOL, RWC, RGR: see Table 1.

Table 3
Mean comparison of physiological traits for the callus of 8 genotypes of safflower

	Cell viability (g^{-1} FW)	INTOL	RWC %	RGR (g g^{-1} FW)	Potassium$^+$	Sodium$^+$	Proline$^+$
G1	9.092^{b}	0.2188^{b}	12.95^{bc}	0.67^{a}	32.36^{d}	7.663^{a}	66.17^{b}
G2	7.797^{c}	0.144^{bc}	9.723^{d}	0.25^{b}	29.07^{e}	6.919^{a}	64.76^{b}
G3	6.587^{d}	0.3403^{a}	13.59^{b}	0.31^{b}	34.15^{c}	6.584^{a}	42.46^{c}
G4	5.832^{e}	0.3037^{a}	12.24^{c}	0.26^{b}	32.55^{d}	8.116^{a}	45.15^{c}
G5	5.956^{e}	0.023^{d}	12.04^{c}	0.57^{a}	42.76^{a}	3.755^{b}	45.36^{c}
G6	11.36^{a}	0.046^{d}	15.85^{a}	-0.19^{c}	34.49^{c}	6.669^{a}	70.62^{a}
G7	5.587^{e}	0.081^{cd}	15.59^{a}	-0.17^{c}	35.17^{c}	4.098^{b}	24.91^{e}
G8	5.762^{e}	0.209^{b}	12.02^{c}	-0.15^{c}	38.99^{b}	4.662^{b}	37.50^{d}

$^+$: µmol g^{-1} d.wt.; Means followed by the same letter(s) in each column are not significantly different (Duncan's Multiple Range Test 5%). INTOL, RWC, RGR: see Table 1.

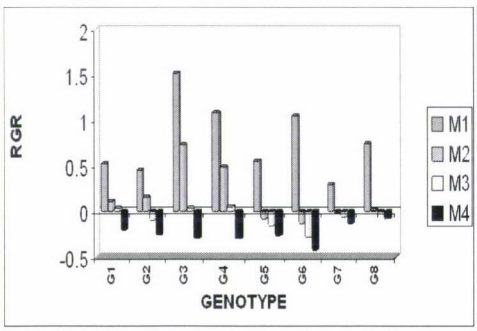

Fig. 1. Comparison of interaction between medium and callus genotype in terms of relative growth rate (RGR). M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol

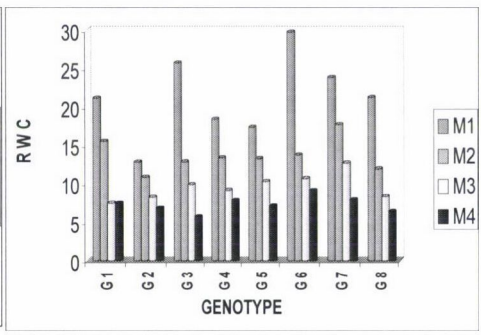

Fig. 2. Comparison of interaction between medium and callus genotype in terms of relative water content (RWC). M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol

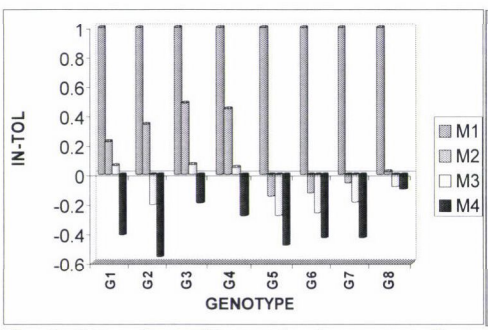

Fig. 3. Comparison of interaction between medium and callus genotype in terms of index of tolerance (INTOL). M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol.

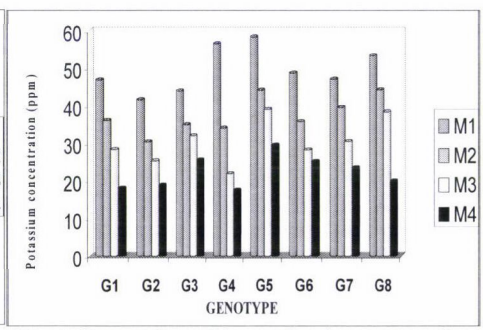

Fig. 4. Comparison of interaction between medium and callus genotype in terms of potassium content. M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol.

K^+ and Na^+ analyses

As shown in Table 2, the Na^+ and K^+ contents declined significantly ($p<0.05$) in hypocotyl-derived calli under mannitol-induced osmotic stress. All the genotypes exhibited a linear decrease in sodium and potassium contents as the concentration of the osmotic stress agent increased (Figs. 4 and 5). The potassium content in G2 (29.07 μmol g^{-1} d.wt.) and the sodium content in G5 (3.755 μmol g^{-1} d.wt.) were lower than in the other genotypes (Table 3). Similar results were obtained for the K^+ content of callus in durum wheat (Almansouri et al., 2000; Lutts et al., 2004), alfalfa and lentil (Yupsanis et al., 2001), *Medicago sativa* L. (Shah et al., 1990) and sugarcane (Errabii et al., 2006). However, contradictory results were published concerning *Oryza sativa* L. (Sajid Aqeel Ahmad et al., 2007) and *Vigna radiata* (Gulati and Jaiwal, 1992). The decrease in K^+ concentration observed in the present work suggests that this cation may not be involved in the osmotic adjustment of the mannitol-treated calli obtained from safflower genotypes. In the case of sodium accumulation, the genotype × drought stress interaction was found to be non-significant (Table 1).

Effect of drought stress on cell viability

The rate of cell viability of calli derived from safflower genotypes under normal and drought stress conditions varied among the cultivars from 5.58 to 11.36, with an average of 7.24 (Table 3, Fig. 6). The cell viability of the G1, G2 and G6 cultivars was higher than the average, while that of G3, G4, G7 and G8 was lower than the average (Table 3). The cell viability decreased significantly when the osmotic stress agent (mannitol) was added (Table 2). A decline in cell viability was reported in callus cultures of sugarcane (Patade et al., 2008), durum wheat (Lutts et al., 2004) and tobacco (Watad et al., 1991) in response to salt stress.

Effect of drought stress on proline accumulation

The free proline contents of calli grown on four concentrations of mannitol (0, 180, 350, 505 mM) were estimated. The free proline content in the calli increased significantly with an increase in mannitol-induced osmotic stress in all the stress regimes (Fig. 7). There was an almost linear increase in proline accumulation with increasing concentrations of mannitol, with the greatest increase in proline content in medium M4 (Table 2). The mean differences in proline content in all eight genotypes were significant at the 0.05 level between all treatments (Table 3). Genotype G6 (Isfahan var.) contained the highest proline concentration (70.62 μmol g^{-1} d.wt) in calli grown under severe drought stress (–1.69 MPa), while the lowest proline level (24.91 μmol g^{-1} d.wt) was measured in the calli of G7 (Hartman var.). It is possible that these differences are due to the over-production of proline in drought-stressed calli and might be due to the decreased activity of proline dehydrogenase, a catabolic enzyme of

proline. Thus, it appears that the increase in proline contents is an adaptive mechanism in safflower. The same result was reported for cotton (Parida et al., 2007). Proline is known to be the most important osmotic-stress marker in *in vitro* systems (Gangopadhyay and Basu, 2000). Its accumulation increases manyfold upon exposure to drought (Barakat and Abdel-Latif, 1995; He and Yu, 1995). The accumulation of proline during drought may have other functions, such as enzyme protection (Solomon et al., 1994) and the stabilization of biological membranes (Van Rensburg et al., 1993), and the degradation of proline may improve the energy status of cells recovering from water deficit (Mattioni et al., 1997).

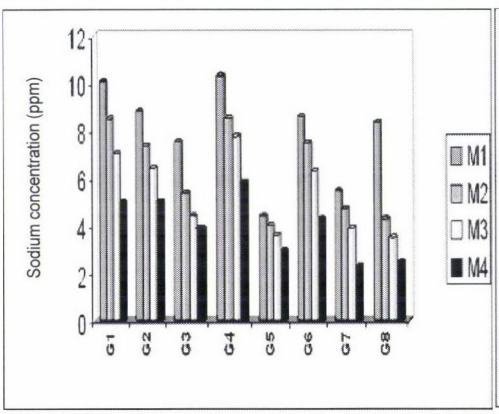

Fig. 5. Comparison of interaction between medium and callus genotype in terms of sodium content. M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol

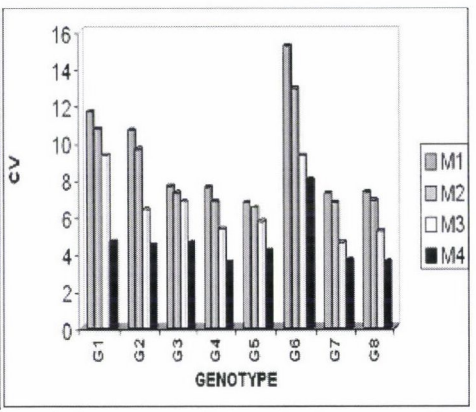

Fig. 6. Comparison of interaction between medium and callus genotype in terms of cell viability (CV). M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol

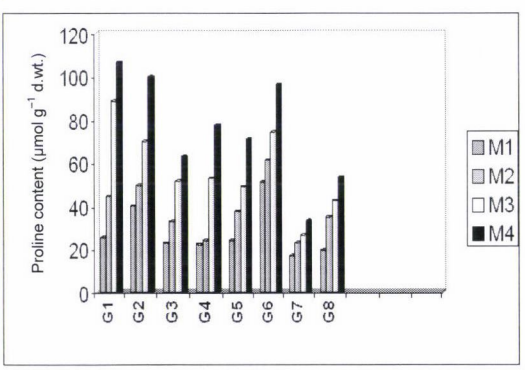

Fig. 7. Comparison of interaction between medium and callus genotype in terms of proline content. M1: medium without mannitol (control), M2: medium containing 180 mM mannitol, M3: medium containing 350 mM mannitol, M4: medium containing 505 mM mannitol

The current results agree with those of other authors, who reported increases in the proline content after drought stress in a number of plant species (Javed and Ikram, 2008; Das et al., 1990; Misra et al., 1996; 2002; Parida et al., 2002; Al-Khayri and Al-Bahrany, 2004; Lopez et al., 1994; Fedina et al., 2002). The reduction in osmotic and water potential may be correlated with the accumulation of solutes such as cations (Almansouri et al., 2000; Javed, 2002), anions (Cl^- and $SO4^{2-}$) (Almansouri et al., 2000; Javed, 2002), proline (Hou et al., 1992; Lutts et al., 1996; Yan et al., 2000; Al-Khayri and Al-Bahrany, 2002), other free amino acids (Paek et al., 1988; Juhasz et al., 1995; 1997; Juhasz and Sarkadi, 1994), soluble proteins (Paek et al., 1988; Hou et al., 1992), carbohydrates (Smith et al., 1984; Almansouri et al., 2000; Javed, 2002) and phenols (Gonzalez and Garcia, 1988). The accumulation of these solutes leads to a reduction in the osmotic potential of the callus tissue at cellular level and hence helps plant to maintain growth in a stressful environment.

To summarize, the results showed that in mannitol-treated calli of safflower, drought induces an increase in proline content and a decrease in RGR, RWC, INTOL, K^+, Na^+ and cell viability. It is suggested that the proline concentration under drought stress could be used as a selection criterion along with the other parameters. The experiments also demonstrated that drought tolerance differs among safflower cultivars. The cultivars Isfahan and LRV-51-51 appeared to be more drought-tolerant under *in vitro* conditions than the other genotypes.

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EFFECT OF CADMIUM-CONTAMINATED SOILS ON DRY MATTER YIELD AND MINERAL COMPOSITION OF RAYA *(Brassica juncea)* AND SPINACH *(Spinacia oleracea)*

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Raya (*Brassica juncea*) and spinach (*Spinacia oleracea*), grown as leafy vegetables, are known to accumulate large amounts of heavy metals in their shoots and roots because of their high biomass and root proliferation. In a pot experiment, a sandy loam soil was polluted with cadmium (Cd) at rates of 0, 5, 10, 20, 40 and 80 mg kg^{-1} soil to assess the accumulation pattern and its effect on the dry matter yield and mineral composition of these vegetables. There was a decrease in dry matter yield due to the phytotoxic effect of Cd. The rate of Cd application at which a significant decline in root and shoot dry matter yield occurred varied depending on the vegetable. It was 20 mg Cd kg^{-1} soil in the shoots for both crops. However, the roots of raya were found to be more tolerant of Cd toxicity than those of spinach, as is evident from the fact that a significant decline in dry matter yield occurred at 20 and 10 mg Cd kg^{-1} soil, respectively. Since no visual toxic symptoms were observed on the leaves of raya in any of the treatments, it is clear that the metal may accumulate in this vegetable without visual evidence of its presence. However, at application levels beyond 40 mg kg^{-1} soil, toxicity symptoms, in the form of interveinal chlorosis of the leaf lamina followed by necrosis and leaf rolling, were clearly evident in the case of spinach. The reduction in root and shoot growth corresponded with the amounts of extractable Cd in the soils. The total content of Cd in the crops increased gradually as the rate of applied Cd rose and the roots accumulated much higher amounts than the shoots. The relationship of Cd with Zn and Fe was synergistic in both roots and shoots at the lower rates, but antagonistic at higher Cd application rates for both the crops, while in the case of Mn and Cu, the relationship was negative and antagonistic.

Key words: cadmium toxicity, raya, spinach, dry matter yield, mineral composition

Introduction

In developing countries like India, the sewage water of big cities is indiscriminately contaminated with untreated industrial effluents and is discharged as such in the sewage system, which is ultimately used for irrigation. The long-term use of such waters results in the accumulation of heavy metals in

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soils, which eventually leads to a higher content of these metals in the crops growing in these soils (Brar et al., 2002; Khurana et al., 2003). Due to their high retention time, these metals remain in the soil in dangerous proportions and thus have implications for human and animal health. Cadmium is one of the most damaging due to its excessive discharge as a byproduct from industries (Mengel et al., 2001). Cadmium is readily absorbed by the roots and translocated to the shoots (Benavides et al., 2005) and is thus frequently accumulated by agriculturally important crops, often exceeding the levels that are toxic to the human or animal population, with a significant potential to impair animal and human health (Sanita di Toppi and Gabrielli, 1999).

In Punjab (India), it is generally leafy vegetables that are grown on sewage-irrigated soils. These are known to accumulate large amounts of heavy metals in their shoots and roots because of their high biomass and root proliferation. Two crops, raya (*Brassica juncea*) and spinach (*Spinacia oleracea*) are often grown on sewage-irrigated soils for human consumption. Because of the low production costs and high productivity per unit, these vegetables are considered highly remunerative and profitable. The two crops can perform well under cold conditions in the winter season.

Materials and methods

A screen-house experiment was conducted on loamy sand soil having a DTPA-extractable Cd content of 0.18 mg kg^{-1} soil to assess the effect of six levels of Cd (0, 5, 10, 20, 40 and 80 mg kg^{-1} soil) in a factorial completely randomized design on the accumulation pattern of raya (*Brassica juncea*) and spinach (*Spinacia oleracea*). Earthern pots were lined with polythene to avoid contamination from the surface of the pot wall and 5 kg of this processed soil was placed in each pot. Each treatment was replicated three times. The pots were kept for one month for the cadmium to attain equilibrium. The crops were sown at field capacity moisture level. The recommended doses of N, P_2O_5 and K_2O were applied at rates of 100, 30 and 30 mg kg^{-1} soil to raya and 87.5, 30 and 30 mg kg^{-1} soil to spinach. The pots were irrigated with deionized water as and when required. Organic carbon, pH and available P and K were determined by standard methods (Page, 1982). The experimental soil was sandy loam with pH 7.98, electrical conductivity 0.34 dS m^{-1}, organic carbon 0.30%, 0.5 M $NaHCO_3$ (pH 8.5)-extractable P 12.8 kg ha^{-1} and 1 N NH_4OAc (pH 7.0)-extractable K 195 kg ha^{-1} soil. The DTPA-extractable Zn, Mn, Fe and Cu contents (Lindsay and Norvell, 1978) were 0.98, 5.40, 12.6 and 0.95 mg kg^{-1} soil, respectively. The crops were harvested after 45 days of growth. The dry matter yield of the shoots and roots of both crops were recorded. After harvest, root and shoot samples were taken from each pot and washed with tap water, dipped in 0.01 N HCl in a plastic tub for a few seconds, and then in distilled and deionised water (Piper, 1960; Tandon, 2003). The samples were first dried in air and then at a temperature of 60°C in a hot-air oven. The dried samples were weighed and ground in a Wiley mill. The samples were digested in a 4:1 di-acid mixture of HNO_3 and $HClO_4$ and the digests were analysed for total Cd and micronutrients using an atomic absorption spectrophotometer (Varian SP2O), with blanks and standards with the following concentration ranges: 0, 0.2, 0.4, 0.8 and 1.2 μg ml^{-1} for Zn and Cd; 0, 0.2, 0.4, 0.8 and 1.0 μg ml^{-1} for Cu; 0, 2.0, 4.0, 6.0, 8.0 and 10.0 μg ml^{-1} for Fe; 0, 1.0, 2.0, 3.0 and 4.0 μg ml^{-1} for Mn. The data were analysed statistically as per the design of the experiment (Panse and Sukhatme, 1967).

Results and discussion

Dry matter yield of shoot and root

There was a gradual reduction in the dry matter yield of the shoots and roots of both the vegetables as the level of Cd was raised from 0 to 80 mg Cd kg^{-1} soil (Table 1). The adverse effect of the added Cd was more marked at the highest rate of application. The rate of Cd application at which the dry matter yield of the roots and shoots significantly declined varied with the vegetable. It was 20 mg Cd kg^{-1} soil in the shoots of both the crops and in the roots of raya and 10 mg Cd kg^{-1} soil for the roots of spinach. It can be seen from Table 1 that the dry matter yield of the shoots and roots decreased from 15.85 to 5.47 and from 5.07 to 1.96 g pot^{-1}, respectively, in raya, and from 14.32 to 4.79 and from 3.86 to 1.30 g pot^{-1} in spinach at the highest rate of applied Cd, i.e. 80 mg Cd kg^{-1} soil. So the yield reduction in response to Cd was of similar proportions in the roots as for the aboveground part of the plants. The reduction in root growth was in accord with the amounts of extractable Cd in the soils. The decrease in yield at the point where the decline became significant, i.e. 20 mg Cd kg^{-1} soil, was 11.7% and 17.1% in the shoots of raya and spinach, respectively, indicating that the magnitude of the decrease varied with the plant species at different Cd levels. The detrimental effect of Cd on the yield was consistent with the results reported by other authors in different crops (Singh and Nayyar, 1991; Dahiya et al., 1991; Narwal et al., 1990; Cieslinski et al., 1996; Bipasha et al., 1997).

Cadmium concentration in roots and shoots

The mean concentration of Cd in the roots and shoots of both the vegetables increased successively with increasing rates of Cd application (Table 2). The mean Cd concentration increased from 2.67 to 158.9 and from 2.50 to 95.8 μg g^{-1} in the roots and shoots of raya at the 80 mg Cd kg^{-1} soil rate of application. Likewise, the mean Cd concentrations in the roots and shoots of spinach were 2.78 and 2.56 μg g^{-1} in the control, increasing to 94.87 and 70.0 μg g^{-1} when the rate of Cd application was raised to 80 mg kg^{-1} soil. A consistently higher concentration of Cd was detected in the roots compared to the shoots. These results are in accordance with the findings of Maclean (1976), Mahler et al. (1982), Narwal et al. (1993) and Khurana (2000). The pattern of Cd accumulation in both the crops varied at different levels of applied Cd. In the control treatment (0 mg Cd kg^{-1} soil) the Cd concentration in the two vegetables did not differ greatly, but at the highest rate of Cd application (80 mg kg^{-1} soil) the Cd concentration in raya was distinctly higher than in spinach. The rate of Cd application at which a significant increase in Cd content was observed varied with the vegetable. An application rate of 10 mg Cd kg^{-1} soil was required to significantly increase the shoot Cd content in spinach, while this was only 5 mg Cd kg^{-1} soil for raya. It is pertinent to note here that although these levels significantly increased the Cd content in the shoots of both raya and spinach, they failed to cause a significant reduction in the dry matter yield of the crops, indicating that Cd is less phytotoxic at these levels.

Table 1
Effect of Cd on the dry matter yield (g pot^{-1}) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	15.85	5.07	14.32	3.86
5	15.57	4.97	14.23	3.66
10	15.05	4.90	13.77	3.49
20	14.00	4.61	11.87	3.32
40	9.25	3.0	7.73	2.22
80	5.47	1.96	4.79	1.30
$SD_{5\%}$	1.97	0.35	1.47	0.36

Table 2
Effect of Cd on the Cd content (μg g^{-1} dry weight) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	2.50	2.67	2.56	2.78
5	8.20	13.55	4.63	7.16
10	17.4	27.85	8.79	14.09
20	32.2	55.79	18.43	32.34
40	62.80	105.29	36.40	52.46
80	95.80	158.87	70.00	94.87
$SD_{5\%}$	2.63	5.49	3.40	3.35

Phytotoxicity values of 5–30 μg g^{-1} (Kabata and Pendias, 1984), 6–70 μg g^{-1} (Singh and Nayyar, 1989; Singh et al., 1989) and 3–20 μg g^{-1} (Singh and Nayyar, 1994) have been reported for Cd for different crops. A significant reduction occurred in the shoots at 20 mg Cd kg^{-1} soil in both raya and spinach, with corresponding Cd concentrations of 32.2 μg Cd g^{-1} dry matter for raya and 18.43 μg Cd g^{-1} dry matter for spinach, which were within the ranges noted by other workers. Further, the absence of phytotoxic symptoms below these values confirmed the fact that contamination of the soil with up to 20 mg Cd kg^{-1} soil did not significantly affect the dry matter yield of either crop. This suggests that provided plants growing in contaminated soil do not take up Cd in amounts exceeding the phototoxic level, the yields as a rule do not decrease. Available reports show marked differences for the Cd tolerance of various plant species, including wheat (Zhang et al., 2002), cotton (Wu et al., 2004), pea (Metwally et al., 2005) and rice (Wu et al., 2006). Cd is readily bio-available and its hyper-accumulation has been reported to cause leaf chlorosis (Baryla et al., 2001; Smeets et al., 2005; Wang and Zhou, 2006).

No visible toxicity symptoms were observed on the leaves of raya up to an application rate of 40 mg kg^{-1} soil, thus illustrating that the metal may accumulate in this vegetable without visual evidence of its presence. However, at application rates beyond 40 mg kg^{-1} soil, toxicity symptoms in the form of interveinal chlorosis of the leaf lamina, followed by necrosis and leaf rolling, were clearly evident in the case of spinach.

Effect of Cd on the Cd uptake (mg pot^{-1}) of shoots and roots

The Cd uptake in raya increased from 40 μg g^{-1} in the control to 128, 264, 451, 581 and 523 μg pot^{-1} at 5, 10, 20, 40 and 80 mg Cd kg^{-1} soil, respectively. Compared with no Cd (control), this represented a 10.3-fold increase in uptake by raya at 20 mg Cd kg^{-1}, suggesting that Cd was readily absorbed by raya and was easily translocated from the roots to aboveground plant parts. Raising the Cd level to 40 mg kg^{-1} soil had a less pronounced effect on the Cd uptake, with an increase only of 0.29 times that recorded at 20 mg Cd kg^{-1} soil. This Cd uptake pattern was the consequence of both the reduction in yield due to Cd toxicity and the increased uptake of Cd at the higher application rate, as the reduction in yield was compensated by higher Cd absorption. The data in Table 3 revealed a similar pattern of Cd uptake for the shoots of spinach. The Cd uptake in the roots of both crops was significantly affected by the level of Cd application. The lower uptake of cadmium in the roots as compared to the shoots, in spite of the high Cd concentration, could be attributed to the lower dry matter yield of the roots. The mean uptake of Cd by the roots increased significantly and progressively with increasing levels of Cd. The increase in mean uptake of Cd in the roots of raya was about 53, 123.4, 243.4, 303.3 and 297.4 mg pot^{-1} at the 5, 10, 20, 40 and 80 mg Cd kg^{-1} soil levels. Lower Cd uptake was recorded in the roots of spinach than in those of raya.

It can thus be concluded that plant species differ widely in their ability to absorb, accumulate and tolerate Cd toxicity. In the present study, raya had higher Cd uptake than spinach. Kuboi et al. (1986) examined the Cd uptake in plant species belonging to nine different families, which could be classified in three groups:

1). Low accumulator: Leguminosae

2). Moderate accumulator: Gramineae, Liliaceae, Cucurbitaceae and Umbelliferae

3). High accumulator: Cruciferae, Chenopodiaceae, Solanaceae and Compositae.

There may thus be specific uptake mechanisms in different plants for heavy metal tolerance. The two crops studied in the present work belong to different botanical families (raya: Cruciferae, spinach: Chenopodiaceae). The higher uptake of Cd by raya indicated that the family Cruciferae was more tolerant than the Chenopodiaceae (spinach). A similar suggestion was put forward by Kumar et al. (1995) and Ebbs and Kochian (1997; 1998), who found that species belonging to the Cruciferae family, such as *Brassica juncea, B. napus* and *B. rapa*, had the potential to accumulate moderate levels of heavy metals and could therefore be used to clean up sites contaminated with toxic metals.

Table 3
Effect of Cd on the Cd uptake (μg pot^{-1}) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	40	13.6	37	11
5	128	67	67	26
10	264	137	121	49
20	451	257	217	107
40	581	317	276	117
80	523	311	333	123
$SD_{5\%}$	99	51	29	9

DTPA-extractable cadmium in post-harvest soils

The content of DTPA-extractable Cd increased in proportion to the rates applied (Table 4). The increase was significant over the control even at a rate of 5 mg Cd kg^{-1} soil. The amount of mean DTPA-extractable Cd in the soil at equilibrium after different rates of Cd application is presented in Table 4.

Compared to its content in equilibrated soil, the DTPA-Cd content in the soil generally decreased after the harvest of both the crops. This could be due both to removal of Cd by the crops and by transformation into relatively insoluble forms. The amount left in the soil after harvest varied with the crop.

Toxic level of Cd in the soil

The toxic or upper critical level of Cd is defined as the lowest tissue or soil concentration at which its presence leads to a reduction in yield. The critical concentration is calculated using an arbitrary level of dry matter yield reduction, usually 10–30%. To find the upper critical level, regression equations were calculated, where the percentage reduction in dry matter yield of each crop was regressed on the corresponding DTPA-Cd content in the soil. From these equations, the toxic level of DTPA-Cd at which a 20% reduction in dry matter yield occurred was then estimated. The toxic levels of DTPA-Cd were found to be 10.28 and 9.00 mg kg^{-1} soil for raya and spinach, respectively, indicating the different tolerance of the crops to cadmium toxicity.

Effect of Cd levels on micronutrient contents (μg g^{-1} dry matter) in raya and spinach

Zinc

It is clear from the data in Table 5 that there was a consistent increase in the mean Zn concentration with increasing levels of Cd up to 20 mg kg^{-1} soil, while the higher rates (40 and 80 mg kg^{-1} soil) depressed the zinc concentration in both the roots and shoots of the crops. This showed that low levels of cadmium have a synergistic effect on the zinc concentration in the shoots and

roots of both crops, while high levels have an antagonistic effect. A significant positive correlation was observed between the rate of Cd application and the Zn concentration of the shoots ($r = 0.91$ for raya and $r = 0.97$ for spinach) and roots ($r = 0.96$ for raya and $r = 0.95$ for spinach) up to the 20 mg Cd kg^{-1} soil rate. At higher rates, however, highly significant negative correlations were obtained for both the shoots ($r = -0.95$ for raya and $r = -0.85$ for spinach) and the roots ($r = -0.95$ for raya and $r = -0.93$ for spinach). The increase in shoot zinc concentration with the application of Cd up to 20 mg kg^{-1} soil may be due to the concentration effect, because the dry matter yields of the shoots and roots were decreased by cadmium. The decrease in zinc content in the plants at higher levels of Cd addition might be due to the competition of these metals for the same absorption sites on the plant root surface, resulting in a lower uptake of zinc, as cadmium and zinc ions have similar charges and possibly use the same carrier sites.

Copper

The copper concentration in the roots and shoots of raya and spinach was negatively affected by the application of Cd at all levels (Table 6). This decrease in the mean Cu concentration in the shoots of raya and spinach became significant at application rates of 10 mg Cd kg^{-1} soil and above. The correlations between these metals were found to be highly negative both in the roots ($r = -0.80$ for raya and $r = -0.91$ for spinach) and in the shoots ($r = -0.84$ for raya and $r = -0.94$ for spinach). This may be ascribed to antagonism between the two elements. The copper content in the roots of the crops was generally higher than in the shoots. These findings are in conformity with the results of Khan and Khan (1983). The antagonistic effect of Cd accumulation on the levels of essential nutrients in the leaves was also observed in chelator-buffered nutrient solution (Adhikari et al., 2006).

Table 4

Effect of Cd on the DTPA-extractable Cd in the soil at equilibrium and after the harvest of raya and spinach

Cd levels (mg kg^{-1} soil)	At equilibrium	After the harvest of raya	After the harvest of spinach
0	0.17	0.08	0.12
5	2.49	2.02	2.35
10	4.65	3.62	4.12
20	8.39	7.25	7.92
40	16.87	14.4	15.42
80	29.63	26.98	28.28
$SD_{5\%}$	0.75	0.64	0.46

Table 5
Effect of Cd on the Zn content (μg g^{-1} dry matter) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	50.2	75.6	38.7	39.9
5	54.7	84.8	40.8	44.0
10	57.1	86.7	45.5	49.0
20	59.1	93.9	48.6	51.9
40	51.5	81.4	41.1	43.8
80	47.2	75.9	34.8	36.4
$SD_{5\%}$	3.0	4.7	2.8	2.7

Table 6
Effect of Cd on the Cu content (μg g^{-1} dry matter) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	11.0	13.7	8.2	11.0
5	10.0	12.4	8.0	10.7
10	8.8	11.6	7.3	10.0
20	8.5	10.8	7.8	9.9
40	8.4	10.4	7.0	9.2
80	8.3	10.1	6.8	8.5
$SD_{5\%}$	1.42	0.80	1.2	0.84

Manganese

There was a consistent decrease in the mean Mn concentration of the shoots of both crops with increasing levels of cadmium, which could be interpreted as a clearcut case of antagonism between Cd and Mn (Table 7). The application of 10 mg Cd kg^{-1} soil significantly decreased the mean Mn concentration in the shoots of raya, but the decrease was non-significant in those of spinach. In the roots, the application of 5 and 10 mg Cd kg^{-1} soil significantly decreased the Mn concentration in both raya and spinach. These findings were corroborated by the negative correlation detected in both the roots ($r = -0.94$ for raya and $r = -0.957$ for spinach) and the shoots ($r = -0.79$ for raya and $r = -0.90$ for spinach). Cataldo et al. (1983) found that pearl millet and green gram showed a depression in the Mn content when 10 and 20 mg Cd kg^{-1} soil was added. These authors attributed this to the fact that Cd competitively inhibited Mn absorption, suggesting a common transport site or process. Patel et al. (1976) also observed that the Mn concentration decreased due to Cd application. On the other hand, Khan and Khan (1983) and Narwal et al. (1993) reported that the Mn concentration increased with the addition of cadmium, in contrast to the findings in the present study.

Table 7
Effect of Cd on the Mn content (μg g^{-1} dry matter) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	76.3	82.0	55.6	59.4
5	71.5	80.1	55.1	60.2
10	62.4	76.7	55.0	59.1
20	51.7	69.5	53.1	57.2
40	54.9	61.8	52.0	55.4
80	48.8	56.8	51.5	53.6
$SD_{5\%}$	4.6	2.8	NS	1.7

NS: Non significant

Iron

The iron concentration in the roots and shoots of both the crops revealed a synergistic relationship between Cd and Fe up to 20 kg^{-1} soil and an antagonistic relationship at higher levels (40 and 80 mg Cd kg^{-1} soil) (Table 8). This is further supported by the high positive correlation between Cd and Fe in the roots ($r = 0.94$ for raya and $r = 0.93$ for spinach up to 20 mg Cd kg^{-1} soil) and shoots ($r = 0.98$ for raya up to 40 mg Cd kg^{-1} soil and $r = 0.95$ for spinach up to 20 mg Cd kg^{-1} soil). The mean Fe concentration in the roots and shoots of both crops showed a decreasing trend beyond an application rate of 40 mg Cd kg^{-1} soil. These findings are in conformity with the results of Khan and Khan (1983), Koshino (1973), Root et al. (1975) and Rupp et al. (1985), who reported similar behaviour in tomato and egg plant, rice, corn and grapevine, respectively. Gupta and Dixit (1992), on the other hand, reported that the Fe content decreased due to the application of Cd in soybean and wheat, while Mahler et al. (1982) found no influence of Cd application on the leaf Fe concentration of lettuce or Swiss chard.

Table 8
Effect of Cd on the Fe content (μg g^{-1} dry matter) in the shoots and roots of raya and spinach

Cd levels (mg kg^{-1} soil)	Raya		Spinach	
	Shoot	Root	Shoot	Root
0	229.8	285.7	445.9	493.4
5	238.7	296.5	451.5	507.7
10	249.9	313.6	462.7	520.4
20	262.5	329.7	470.5	521.4
40	259.2	328.1	461.7	503.7
80	212.5	278.3	437.9	484.9
$SD_{5\%}$	12.8	11.1	10.8	10.7

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EFFECT OF AGRO-ECOSYSTEM COMPONENTS ON THE POPULATION DYNAMICS OF EUROPEAN BROWN HARE (*Lepus europaeus* Pallas)

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Most Hungarian ecosystems are agro-ecosystems dominated by crops. In these agro-ecosystems small game has a secondary, but nevertheless very important role. On the one hand, the wild fauna figures are an indicator of the sustainability of a system. On the other hand, game management and hunting are traditional activities. Hunting can be regarded as a special kind of agriculture and small game management takes place on agricultural land, so more should be known about the connection between ecosystem components, such as climatic factors, crops and small game populations, for example that of hare.

Data on the land use in Hungary were collected between 1960 and 2006. The area and yield of 30 field crops and various meteorological parameters were examined, as well as hare populations.

The results suggest that in general the magnitude of crop areas had a stronger effect than the yield, while weather parameters had the weakest impact on the hare population.

Key words: agro-ecology, European brown hare (*Lepus europaeus*), crops, land use

Introduction

Sustainability is a complex term, which can be defined in many ways, but the essence of sustainable development is economic development that is in harmony with the regeneration of natural resources and the assimilation of stresses in the environment (Brown et al., 2000). The management and conservation of natural resources and the orientation of technological and institutional changes should ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable development (in agriculture, fisheries and forestry) conserves land, water, and plant and animal genetic resources, and is environmentally non-degrading, technically appropriate, economically viable and socially acceptable (Láng, 2004).

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In addition to the regeneration of natural resources and the assimilation of environmental stress, sustainable agriculture should also protect human health and improve living standards and the carrying capacity of rural areas (Győrffy, 1993; Várallyay et al., 1985). In agricultural and rural areas, the wild fauna population is an indicator of the sustainability of a given system. All human activities, especially farming, may have an impact on agro-ecosystems, so good agricultural practice is essential for maintaining sustainability (Jolánkai and Németh, 2002; Szöllősi et al., 2004).

In Hungary, more than half of the total area is used by agriculture, so one of the most important kinds of ecosystems is the agro-ecosystem. Each specific ecosystem contains not only the crops that are sown, but many other organisms as well. From the plant protection point of view, these include pests, fungi, weeds, etc., but other organisms are neutral (or almost neutral) for the crops in the agro-ecosystem. Animals that originally lived on open (non-forest) land adapted to living on agricultural areas when the land was taken over for farming.

These species include not only protected and rare ones, but others that can be hunted. Game management should be considered as a part of agriculture; small game management is a special kind of land use (Faragó, 2002; Csányi, 2007).

One of the most important small game species in Hungary is the European brown hare (*Lepus europaeus* Pallas). Unfortunately, in recent decades the hare population of Hungary has decreased, similarly to other European populations. There has been much speculation as to the causes, but detailed studies on all the components of agro-ecosystems and their interactions will be required if hare populations, and the agro-ecosystem they are part of, are to be saved. These should include a profound analysis of agronomic, meteorological and wildlife statistical data.

In the present study, data on the land use in Hungary were collected over the period 1960 to 2006. The area and yield of 30 field crops and various meteorological parameters were examined, together with hare population figures.

Materials and methods

In an average population, the dynamics are formed by migration, birth and death (Csányi, 2007). If N_1 is the number of individuals at time 1 then:

$$N_1 = N_0 + B - D + I - E$$

where N_0 is the number of individuals at time 0, B is the number of individuals born, D the number that died, I the number that immigrated, and E the number that emigrated between time 0 and time 1.

In the case of hares, it was found that migration was not typical (Angelici et al., 1999), so the equation can be simplified:

$$N_1 = N_0 + B - D$$

It is important to note that the number of births seems to be constant over a given period of the year, in a given geographical area (Blottner et al., 2001; Hacklander et al., 2001). In Hungary 9 leverets can be expected per mother per year, with a sex ratio of about 1:1 and there is no

reproduction in the first year (Kovács and Heltay, 1993; Faragó, 2002). This can be expressed for each year as:

$$B = (N_0/2) \times 9$$

Combining the two equations:

$$N_1 = N_0 + [(N_0/2) \times 9] - D$$

or

$$N_1 = 5.5 \times N_0 - D$$

The death rate for each year can be calculated from the total number of hares as:

$$D = 5.5 \times N_0 - N_1$$

As the death rate includes exploitation by hunting, this is a central component for management. Successful management of the hare population will also require a knowledge of the cause of death. As data on hunting are available, it is possible to use the equation:

$$D = H + M$$

where H is the number exploited by hunting and M is the mortality. Combining the equations:

$$H + M = 5.5 \times N_0 - N_1$$

or

$$M = 5.5 \times N_0 - N_1 - H$$

As the other components of the equation are countable, it is possible to calculate the mortality, which is caused mostly by environmental factors that are part of the agro-ecosystem. Statistics are available for many of these factors, such as the area of each crop, the yield of each crop, the weather, etc. The size of the hare population and the number of hares hunted each year are also found in databases.

The land use categories considered were: arable land, forest, grassland, agricultural land, reed, fishpond, cultivated area, set aside, horticulture, orchard, total area.

The main crops were: wheat, maize, barley, rye, oats, rape, sunflower, sugar beet, potato, hemp, flax, poppy, tobacco, dry peas, soybean, bean, green peas, lentil, alfalfa, red clover, silage maize, spring and winter green forage mixtures, onion, cabbage, tomato, watermelon, melon, green paprika, red pepper, vine.

The following weather parameters were recorded: average annual temperature, highest daily temperature in the year, lowest daily temperature in the year, highest mean temperature in the year, yearly average of daily maximum temperatures, days above 25°C, days above 30°C, days above 35°C, lowest temperature in the year, yearly average of daily minimum temperatures, days below 0°C, days below –10°C, days below –20°C, yearly total precipitation, yearly precipitation as snow, maximal daily precipitation, daily precipitation above 0.1 mm, daily precipitation above 1 mm, daily precipitation above 5 mm, daily precipitation above 10 mm, daily precipitation above 20 mm, daily precipitation above 30 mm, daily precipitation above 50 mm, days with snow, frost, rain storms or sleet, total yearly duration of sunlight, maximal daily sunlight radiation, days with sunlight duration less that 20% or more than 80% of the maximum.

If statistical correlations are found between any of the parameters of the agro-ecosystem and hare mortality, these could be useful for hare management. However, it is necessary to know how strong the correlation is, what change in the hare population is caused by unit change in the parameter, and how changeable the parameter is.

Statistical analysis was performed using the MS Excel programme packages. The mortality for each year was assigned to the value of each parameter in the same year, resulting in 60 points indicating the correlations between each parameter and hare mortality. These points were fitted to give a trend-line and to calculate Pearson's correlation coefficient, r^2. The slope of the trend-line revealed whether a change in the given parameter caused a fast or slow change in the hare population dynamics. The difference between the maximum and minimum values of each component in the agro-ecological system and its relation to its maximum indicated the variability of the studied parameter.

A steeper trend-line means a faster change in the hare population, while a trend-line closer to the ordinate axis indicates greater variability. These two parameters can be used to describe the connection between hare mortality and each component of the agro-ecosystem, but first they must be standardized. The value of the slope can be expressed as a percentage using the difference between the biggest and smallest slopes as a reference and making correlations between this reference value and each slope value (if the slope is negative, the number will also be negative). The other parameter describing the trend lines (r^2) is already a percentage. If these values are all reduced by 50, the lower ones will be in the range –50 to 0 and the higher ones in the range 0 to 50.

The relationship between hare mortality and each component of the agro-ecosystem can thus be described by two parameters. A clearer picture can be obtained by forming groups on the basis of the closeness of the correlation. Parameters for each component can be displayed in a coordinate system, where each quadrant can be interpreted as follows:

Quadrant I (+;+): the agro-ecosystem component is variable and causes a major change in the hare population

Quadrant II (–;+): the agro-ecosystem component is less variable and causes a major change in the hare population

Quadrant III (–;–): the agro-ecosystem component is less variable and causes a minor change in the hare population

Quadrant IV (+;–): the agro-ecosystem component is variable and causes a minor change in the hare population.

Results

The data were used to compile a series of points from which the indices described above were calculated. After calculating the correlation coefficients (r^2), the results were grouped according to the closeness of the correlation in the following intervals: >0.71, 0.651–0.7, 0.51–0.65, 0.41–0.5, <0.4.

The results for the first group are illustrated in Figure 1. The other groups contained far more components, so the differences can be seen more clearly in tables (Tables 1, 2, 3 and 4).

The remaining 77 components (22 referring to area, 23 to yield and 32 to weather parameters) can be found in the last group, with the loosest correlation (r^2<0.4). In this group 35 elements are located in quadrant I, 7 in quadrant II, 6 in quadrant III and 29 in quadrant IV.

In the strongest category (r^2>0.71), although two parameters have fairly high slope, they have very low variability. The size of the hare population has a strong positive correlation with the area of arable land and a strong negative correlation with the area of forests. The other parameters have no real effect on the hare population; the strong correlation may be attributed to the fact that they change due to the same causes as hare populations.

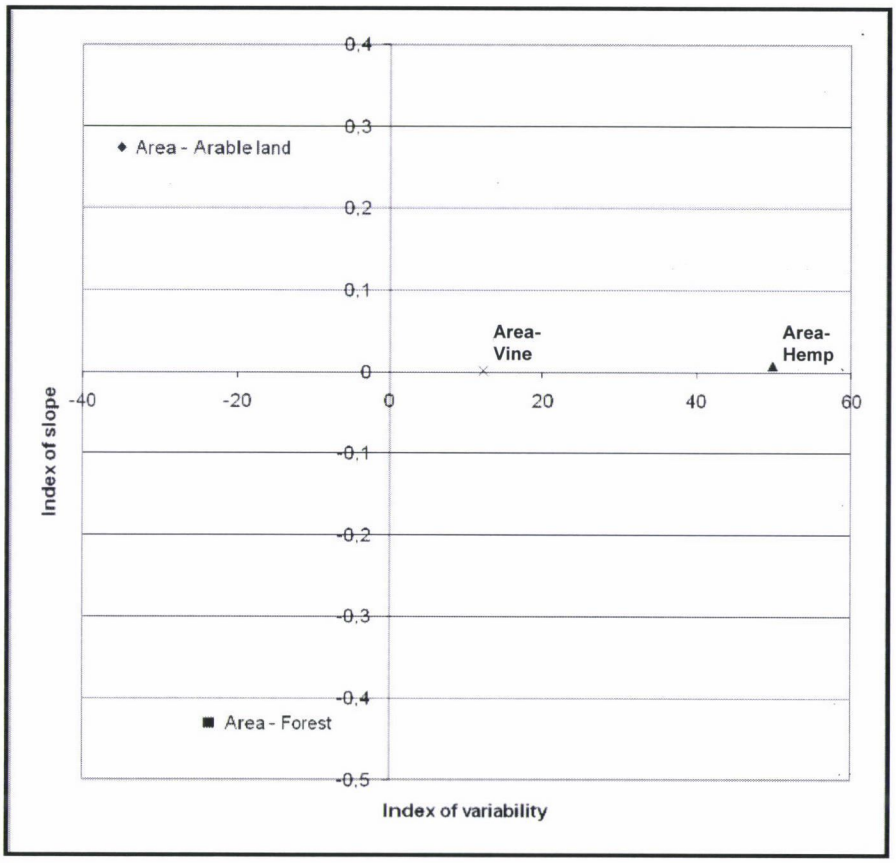

Fig. 1. Spatial distribution of results in the first group (r^2>0.71)

Table 1
Results in the first group (r^2>0.71) and the quadrant each component is located in

Agro-ecosystem component	Index of variability	Index of slope	Quadrant
Area – arable land	–34.74518814	0.27393638	II
Area – forest	–23.51832048	–0.430069314	III
Area – hemp	49.70706856	0.00768396	I
Area – vine	12.19716936	0.001221341	I

Table 2
Results in the second group (r^2=0.651–0.7) and the quadrant each component is located in

Agro-ecosystem component	Index of variability	Index of slope	Quadrant
Area – viticulture	15.12570965	1.129027888	I
Area – grassland	–19.5196327	0.543178888	II
Area – agricultural land	–31.34461077	0.14627813	II
Area – potato	40.48896804	0.000870807	I
Area – sunflower	37.14045466	–0.000365873	IV

Table 3
Results in the third group (r^2=0.51–0.65) and the quadrant each component is located in

Agro-ecosystem component	Index of variability	Index of slope	Quadrant
Area – reed	7.903225806	–4.837724585	IV
Area – fishpond	–17.54385965	–10.1830492	III
Area – cultivated area	–40.64720183	0.211452086	II
Area – set aside	–1.137134052	–0.211466888	III
Area – rye	36.76485236	0.000780449	I
Area – tobacco	26.13355503	0.01043616	I
Area – flax	50.00000000	0.013926003	I
Area – red clover	45.88178267	0.001270578	I
Area – melon	43.75140988	0.047509976	I
Yield – rye	13.63636364	–0.098928545	IV
Yield – potato	20.95948827	–0.01192266	IV
Yield – sunflower	18.01619433	–0.114998671	IV
Yield – grape	28.5942492	–0.032197572	IV

Table 4
Results in the fourth group (r^2=0.41–0.5) and the quadrant each component is located in

Agro-ecosystem component	Index of variability	Index of slope	Quadrant
Area – rape	48.3555629	–0.00109693	IV
Area – alfalfa	15.4955249	0.000531279	I
Area – tomato	34.40282302	0.010852615	I
Yield – wheat	21.19266055	–0.041046874	IV
Yield – maize	23.14814815	–0.03086425	IV
Yield – tobacco	10.8490566	–0.138880636	IV
Yield – onion	19.50517837	–0.010619958	IV

The second strongest category (r^2=0.651–0.7) contains two items, the area of viticulture and potato, in quadrant I of the coordinate system. The other three, grassland, agricultural land and sunflower, have relatively high slope but they are not so variable.

In the next category (r^2=0.51–0.65) most of the items have no real effect on the hare population, while two have a considerable negative effect, as hares do not use fishponds or reeds as a habitat.

The fourth category (r^2=0.41–0.5) included several yield data, all of which have a negative effect on the hare population.

In the last category the closeness of the correlation is low (r^2<0.4). All the weather data were found in this group. The temperature, especially extreme values, has a relatively high effect, which is mostly negative.

In general it can be seen that the magnitude of crop areas has a greater effect than the yield, while weather parameters have the weakest impact.

Discussion

It could be seen from the results that very few of the items studied were strongly correlated with hare populations, so quadrant I was mostly empty. However, an increase in the area of crops or land use types that are favourable for hares had a positive effect on the hare population, while those that are unfavourable had a negative effect.

An increase in crop yields had either no effect or a negative effect on the hare population. As hares are able to find enough food on any kind of crop field, higher yields do not have a positive effect, while more intense farming activities disturb the hares. Rising yields are not beneficial, as food shortages arise during certain periods of the year, not due to overpopulation in a given field.

The weather also had a very weak correlation with the hare population. As the hare is a native member of ecosystems in the Carpathian Basin, only very extreme conditions are likely to influence the population dynamics. The idea that drops in the hare population are due to the weather is unscientific and is confuted by the present results.

The component of the agro-ecological system that exhibited the strongest correlation with hare population dynamics was the area of agricultural crops, though some crops had a positive and some a negative effect, and the closeness of the correlation also varied. If the population dynamics of the brown hare is to be changed, the area of crops and land use methods beneficial for hares should be raised and unfavourable ones should be avoided. Further factors should be examined, alone and in combination, to provide a more precise picture.

In this study the hare population was used as an indicator of the health of the given agro-ecosystem. If changes in the habitat lead to a growth of the hare population, it can be said that the health of the whole agro-ecosystem will increase, which is a very important goal of sustainable agriculture.

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Review

APPLICATION OF GENETIC ENGINEERING IN POTATO BREEDING

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Potato breeding programmes worldwide are undergoing a period of rapid change. In order to be successful, breeders must adapt and incorporate the newest up-to-date techniques as they become available. Recent advances in biotechnology make it possible to develop and cultivate more and more sophisticated transgenic crops with multiple modified traits. Gene transfer methods can be used for a wide range of fundamental studies, contributing to a better understanding of the mechanisms of plant/pathogen interactions and the metabolic pathways in plants. Transgenic potato plants are being generated worldwide to investigate the impact of transgene expression on parameters as complex as yield. Historically, potato was one of the first successfully transformed crop plants. Nowadays, transgenic potatoes have been introduced into the food chain of people and animals in several countries. Some of the genetic modifications give potato plants increased resistance to biotic and abiotic environmental factors, while others lead to improved nutritional value, or cause the plants to produce proteins of the immune system of humans or animals or substances that may be used as vaccines in humans or veterinary medicine. The trend today is towards the generation of crops with output traits, e.g. modified starch or carotenoids, or the production of pharmaceuticals in tubers, whereas the early targets were input traits, e.g. herbicide resistance, pest or virus resistance. This review provides a summary of examples illustrating the versatility and applicability of transgenic biology in potato improvement.

Key words: potato breeding, genetic engineering, GMO

Introduction

During the last decade genetically modified organisms (GMOs) whose genetic information has been changed by genetic engineering have become a reality in our lives. The transformed traits of the genetically modified (GM) crops currently cultivated can be generalized as 'first-generation' targets, largely based on single-gene modification. This is due to the substantial time-lag from the laboratory to field-scale commercial cultivation. Recent rapid developments

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in technology may allow much more sophisticated and complex transgenic crops to be grown in the future (Vreugdenhil et al., 2007). One of the key requirements for an efficient transformation system is the ability to readily regenerate plants from an isolated explant, and in potato a number of strategies have been successfully employed for a wide range of germplasm (Wheeler et al., 1985).

In the sphere of genetic modifications, potato represents a suitable model plant for several reasons. Its undisputable advantage, in contrast to other model plants, is the presence of tubers, which serve as a source of carbon and nitrogen. Moreover, potato reproduces vegetatively, and thus a particular feature may be conserved for a long time. On the other hand, its drawbacks include the tetraploid genome, the low numbers of mutants produced and the relatively high genetic variability (Davies, 1996).

Indeed, historically, potato was one of the first of over 120 plant species that have been successfully transformed with a wide and increasingly sophisticated range of traits (Ooms et al., 1986). The early targets were input traits, e.g. herbicide resistance (Eberlein et al., 1988), pest resistance (Cheng et al., 1992) and virus resistance (Kawchuk et al., 1991). In the last decade, genetic transformation has repeatedly been used in order to improve characters such as resistance to fungi, bacteria and viruses or to modify protein or starch content (Chachulska et al., 1997; Flis and Zimnoch-Guzowska, 2000; Gazendam et al., 2004; Missiou et al., 2004; Prescha et al., 2002; Zuk et al., 2003; 2005). The trend today is towards the generation of crops with output traits, e.g. modified starch (Vardy et al., 2002), carotenoids (Laurence et al., 2005) and the production of pharmaceuticals in tubers (Park and Cheong, 2002).

Modification of photosynthetic performance and tuber development

A range of environmental factors such as light regulate plant growth, development and metabolic activities. Over-expressing one of the photoreceptors that control development processes, such as germination, photomorphogenesis, flowering and senescence, or metabolic processes, such as photosynthesis and assimilate allocation, might enhance the agricultural productivity of crop plants (Smith, 1992). Phytochromes are promising candidates for such an improvement (Robson et al., 1996).

Transgenic potato (*Solanum tuberosum*) plants expressing *Arabidopsis* phytochrome B were characterized morphologically and physiologically under white light in a greenhouse to explore their potential for improved photosynthesis and higher tuber yield. As expected, the overexpression of functional phytochrome B caused pleiotropic effects such as semidwarfism, decreased apical dominance, a higher number of smaller but thicker leaves, and increased pigmentation. Because of the increased numbers of chloroplasts in elongated palisade cells, the photosynthesis per leaf area and in each individual plant increased. In addition, photosynthesis was less sensitive to photoinactivation under prolonged light stress. The beginning of senescence was

not delayed, but the deceleration of chlorophyll degradation extended the lifetime of photosynthetically active plants. Both the higher photosynthetic performance and the longer lifespan of the transgenic plants allowed greater biomass production, including increased tuber yield (Thiele et al., 1999).

Increasing resistance to viruses (by coat protein gene)

Viruses are major yield- and quality-limiting factors in susceptible potato cultivars. In addition to the commercial use of natural resistance genes from wild *Solanum* species in potato breeding programmes, several new approaches, ideas and technologies have emerged recently that could affect the future direction of virus resistance breeding. The practical application of pathogen-derived transgenic resistance has arrived with the first release of GM potatoes engineered for virus resistance in the USA. Transformation with viral coat protein genes was the first approach adopted for pathogen-derived resistance, because it was thought it might work like cross-protection (Pierpoint, 1996). Transformation with the coat protein gene of Potato virus X (PVX) was one of the first attempts to obtain pathogen-derived resistance to a major potato virus (Hermenway et al., 1998). In the Netherlands, too, the first genetically modified potatoes were virus-resistant potatoes. Transgenic potatoes with resistance to PVX were subjected to cultivation tests and will be ready for commercial introduction within a few years, as reported by Bijman (2000). Resistance to potato leafroll virus (PLRV) was also investigated after insertion of the coat protein gene of PLRV into the genome of potato. Although a detectable level of coat protein was not accumulated in any of the tested plants, virus-infected transgenic plants contained markedly lower levels of viral antigen than control plants; this resulted from a reduced rate of virus multiplication in the transgenic plants (van der Wilk et al., 1991). For the introduction of resistance to Potato virus Y (PVY), coat protein gene-induced resistance was applied in several cases (Beachy, 1997; Józsa et al., 2002). Mixed infection of potato plants by different viruses frequently occurs under natural conditions. PVX and PVY infection in potato may result in severe loss in the certification of seed potatoes and affect quality and yield in commercial production. Lawson et al. (1990) transformed a major commercial cultivar, Russet Burbank, with the coat protein genes of PVX and PVY. Transgenic plants that expressed both CP genes were resistant to infection by both the viruses after mechanical inoculation. One line was also resistant when PVY was inoculated with viruliferous green peach aphids.

Increasing resistance to fungi and bacteria

The most serious fungal disease of potato is the late blight caused by *Phytophthora infestans*. Some consider this to be the most dangerous plant disease of all, because it can spread extremely rapidly when conditions are warm and moist, leading to devastating losses. Owing to its flexibility, the disease has been able to survive every management strategy used thus far and has responded

with new, adapted forms. Today, the disease is combated using fungicides and heavy metal treatments. In the meantime, genetic engineers have come up with a promising new strategy.

It may be possible to control fungal infection in potato plants by generating transgenic plants carrying gene *ac2* from amaranth, *Amaranthus caudatus* (Lipkova et al., 2001). The expression of this gene results in a protein which is highly homologous to the cysteine/glycine-rich domains in the chitin-binding proteins (Broekaert et al., 1992). The binding of these to the chitin localized in internal fungal cell walls caused an alteration in their polarity and finally inhibited the growth of the fungi (Selitrennikoff, 2001).

Studies indicated that chitinases are one of the major classes of pathogenesis-related (PR) proteins in plants, which are believed to play important roles in plant defence against infection by pathogens (Melchers et al., 1994; Neuhaus, 1999). Chye et al. (2005) reported the evaluation of potato lines transformed with the *Brassica juncea* chitinase and *Hevea brasiliensis* beta-1,3-glucanase genes. They demonstrated that young transgenic potato plants co-expressing either or both of the genes showed healthier root development than untransformed plants in soil infected with *Rhizoctonia solani.*

Temporin A is a small, naturally occurring antimicrobial peptide, which enhances plant resistance not only to potato blight, but also to wet rot of bacterial origin. This is a disease caused by the fungus *Phytophthora erythroseptica* and the bacterium *Erwinia carotovora.* The results confirmed that transgenic potato plants that express temporin A can serve as a good tool for the control of the most significant fungal pathogens such as *P. infestans* and *P. erythroseptica* (Osusky et al., 2004).

The over-production of hydrogen peroxide in plants is another approach to defend plants against pathogens. In the presence of molecular oxygen, glucose oxidase catalyses ß-D-glucose oxidation, releasing gluconic acid and hydrogen peroxide. The glucose oxidase gene from *Aspergillus niger* was tested from this aspect. Potato plants that produce hydrogen peroxide were characterized by increased resistance to potato blight (*P. infestans*) and to bacterial rot caused by *Erwinia carotovora* (Wu et al., 1995).

Increasing resistance to pests

Potato beetle (*Leptinotarsa decemlineata*) is one of the most important potato plant pests, which often becomes resistant to chemical insecticides. GM potatoes carrying gene *Cry3A*, originating from the soil bacterium *Bacillus thuringiensis* (Bt), were produced to control this beetle. The toxic protein produced by this gene is present in the leaves; after ingestion by the potato beetle, it passes into the intestines and causes the death of the pest. This protein affects all the developmental stages of potato beetles in the same way, but does not affect their natural enemies (Perlak et al., 1993). Several GM potato cultivars with improved resistance to the potato beetle have been approved in the US and

in Canada (Côté et al., 2005; Romeis et al., 2008). Potatoes carrying genes for the production of other insecticide proteins have also been developed, such as snowdrop (*Galanthus nivalis*) lectins (GNA), wheat α-amylase inhibitors (WAI) and bean chitinases (BCH). The insecticidal capability of these transgenic plants was tested in peach-potato aphid (*Myzus persicum*). The best insecticidal effect was recorded for genes that code the lectins (GNA) from snowdrop. In a subsequent study the influence of transgenic potato plants expressing the above-mentioned proteins in the larvae of the moth *Lacanobia olearacea* was tested. All the plants expressing GNA showed an enhanced level of resistance. These results support the hypothesis that GNA has a significant adverse effect on insects (Gatehouse et al., 1996; 1997).

The potato tuber moth, PTM, *Phthorimaea operculella* (Zeller), is one of the most damaging potato pests in tropical and subtropical areas, while *Symmetrischema tangolias* (Gyen), another PTM species, is a serious potato pest in the Andean region. Damage is often observed on potato foliage, stems and tubers. The Bt strategy has proved to be effective in reducing PTM infestations in stores. The expression of the Bt genes confers non-conventional host plant resistance to this pest. Lagnaoui et al. (2000) evaluated the effect of Bt-cry1Ia1 (cryV, now designated cry1Ia1 under the revised nomenclature) transgenic potato plants on the two species of potato tuber moth mentioned above. Detached leaf bioassays were done using 10 neonate larvae per replication on each transgenic line of the potato varieties Atlantic and Spunta. The mortality in Atlantic transgenic plants was lower for *P. operculella* (ranging from 18 to 34%), than for *S. tangolias* (ranging from 40 to 94%). All the transformed Spunta lines tested showed high levels of mortality in both species, with mortality ranging between 80 and 98%. The results of both PTM bioassays demonstrated that high levels of Bt-cry1Ia1 expression can be achieved with the gene construct and vectors used in Spunta transgenic lines. The Bt-cry1Ia1 gene offers another source of resistance that can be pyramided for the effective development of durable resistance to PTM and other insect pest.

Increasing resistance to herbicides

One of the major challenges for potato farmers is the necessary reduction of pesticide use. Besides genotypes resistant to insect pests and microbial pathogens, lines resistant to herbicides have also been generated. The validity of such modifications consists above all in the potential application of a herbicide in the most suitable period with the concurrent maximum reduction in weeds (Slater et al., 2003). The insertion of the bar gene (PAT) from the bacterium *Streptomyces hygroscopicus* into potato plants is an example. Modified potato plants proved to be resistant to the herbicide phosphinothricin (Padegimas et al., 1994).

Transgenic plants expressing various species of the detoxifying enzyme, cytochrome P450 monooxygenase from mammals, were bred by *Agrobacterium-*

mediated transformation. Cytochrome P450 (or CYP) enzymes are involved in the metabolism of herbicides in plants. P450, in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase), catalyses the oxidation reactions of lipophilic compounds, including herbicides. These P450 species play an important role in herbicide selectivity and resistance (Werck-Reichhart et al., 2000). Eleven P450 species in the human liver have been reported to be involved in more than 90% of the P450-dependent metabolism of drugs (Funae et al., 1998). Ohkawa and Ohkawa (2002) examined microsomes from recombinant yeast strains expressing each of the 11 human P450 species. Three transgenic potato lines expressing CYP1A1, CYP2B6 and CYP2C19 exhibited higher cross-tolerance to herbicides with various modes of action and chemical structures. It was concluded that transgenic potato plants with P450s that showed cross-tolerance could be expected to tolerate different herbicides in rotation. A combination of the transgenic plants and the rotation of different herbicides will help prevent the emergence of mutant weeds tolerant to herbicides.

Increasing resistance to abiotic stresses

Due to the continuous thinning of the ozone layer and climatic changes related to global warming, one of the major biotechnological aims is to produce stress-resistant plants (Slater et al., 2003). Free radicals and oxidative stress result from stress factors related to high soil salt level, ambient temperature and water availability. Potato plants resistant to increased soil salt content were produced by inserting a gene for glyceraldehyde-3-phosphate-dehydrogenase (GPD) from oyster mushroom (*Pleurotus sajor-caju*). The effect of the protein was tested by cultivating potato plants in soil containing sodium chloride. Whereas GPD-free potato plants died within a few days, transgenic plants exhibited high tolerance to the presence of salt (Jeong et al., 2001).

In the case of ambient temperature, all plants are sensitive to damage caused by cold and frost. When the ambient temperature decreases (–1°C), ice crystals are formed in the extracellular matrix. This causes dehydration of the cytoplasm, with subsequent shrinkage of membranes. Electrolytes are concurrently released from damaged cells. Potato plants resistant to cold and frost were produced by means of a synthetic gene for AFP protein, derived from winter flounder (*Pseudopleuronectes americanus*). The generated transgenic plants were more resistant to frost-caused damage, and lost fewer electrolytes at low temperatures in comparison with control plants. Resistance to frost was directly correlated with the rate of AFP protein expression in the plant leaves. The validity of this modification consists not only in the possibility of cultivating commercial crops in different geographical zones, but also in the prolongation of the vegetation period (Wallis et al., 1997).

Oxygen deprivation, either partial (hypoxia) or complete (anoxia), restricts ATP production through oxidative phosphorylation and consequently inhibits energy-dependent processes. Most plants cannot survive long periods of

low oxygen, which causes tissue injury and yield loss (Albrecht et al., 2004;Crawford and Breandle, 1996; Drew, 1997; Mustroph and Albrecht, 2003; Perata and Alpi, 1993; Schlueter and Crawford, 2001; Vartapetian and Jackson, 1997). Plants may utilize pyrophosphate (PPi) as an alternative energy donor during ATP deficiency (Stitt, 1998). PPi content was found to be relatively stable under hypoxic conditions (Dancer and ap Rees, 1989; Geigenberger et al., 2000; Mohanty et al., 1993). Mustroph et al. (2005) revealed that low levels of pyrophosphate in transgenic potato plants expressing *E. coli* pyrophosphatase lead to decreased vitality under oxygen deficiency. UDP glucose was found to accumulate in the roots of transgenic plants containing 40% less PPi, while the concentrations of hexose-6-phosphate, other glycolytic intermediates and ATP decreased, leading to growth retardation under aerated conditions. Apart from metabolic alteration, the activity of sucrose synthase was increased to a lower extent in the transgenic line than in the wild type during hypoxia. These data suggest that sucrose cleavage was inhibited due to PPi deficiency even under aerated conditions, which has severe consequences for plant vitality under low oxygen. This is indicated by a reduction in the glycolytic activity, lower ATP levels and an impaired ability to resume growth after 4 d of hypoxia. The phosphorylation of fructose-6-phosphate via PPi-dependent phosphofructokinase was not altered in the roots of transgenic plants. Nevertheless, these data provide some evidence for the importance of PPi in maintaining plant growth and metabolism under oxygen deprivation.

Modification of internal content and increasing nutritive value of tubers

Amino acid content

The improvement of the nutritive value of crop plants, in particular the amino acid composition, has been a major long-term goal of plant breeding programmes. The essential amino acids that limit the nutritive value of potato protein are lysine, tyrosine and the sulphur-containing amino acids methionine and cysteine (Jaynes et al., 1986). Chakraborty et al. (2000) reported the cloning of a gene that encodes a seed-specific protein, amaranth seed albumin (AmA1), from *Amaranthus hypochondriacus* (Raina and Datta, 1992). The AmA1 protein is non allergenic in nature and is rich in all essential amino acids, while its composition corresponds well with the World Health Organization standards for optimal human nutrition. In an attempt to improve the nutritional value of potato the AmA1 coding sequence was successfully introduced and expressed in a tuber-specific and constitutive manner in potato. There was a striking increase in the growth and production of tubers in transgenic plants and also in the total protein content, with an increase in most essential amino acids. The expressed protein was localized in the cytoplasm as well as in the vacuole of transgenic tubers (Chakraborty et al., 2000)

Sugar content

GM potatoes with an inserted phosphofructokinase gene from the bacterium *Lactobacillus bulgaricus* were produced by Czech researchers for the modification of tuber sugar content. This gene causes the degradation of simple sugars via the glycolytic pathway. Despite the fact that potato plants contain their own phosphofructokinase, there is one substantial difference. In contrast to the bacterial enzyme, the potato plant enzyme is cold-sensitive and consequently does not function at lower temperatures. This causes problems during potato storage at low temperatures, as they become sweet due to the accumulation of simple sugars. Moreover, potatoes containing higher amounts of simple sugars turn brown during frying and are consequently less attractive for consumers. Transgenic potato plants not only have lower sugar content, but the chips prepared from such potatoes were lighter in colour than those prepared from non-modified ones (Navratil et al., 1998).

Starch form

Potatoes are becoming more and more important as renewable raw materials for the starch industry. For starch potatoes, taste is not important. Instead, emphasis was placed on the quality and composition of the starch. Potato starch is composed of a mixture of amylose and amylopectin. These two kinds of starch have very different properties from the industrial point of view. The mixture of different starches is a problem, because they have to be separated using expensive processes that also take a toll on the environment. At present emphasis has been placed upon developing potatoes containing only amylopectin, due to its diverse applications. Classical breeding methods have not yet been able to provide an amylose-free potato that has acceptable yield and resistance to pests and diseases. Genetic engineering (antisense strategy), on the other hand, offers a targeted approach to suppressing the production of amylose. Genetically modified amylopectin potatoes have been tested in field trials for several years and recently Amflora, a new GM potato variety, was released for cultivation in the EU, becoming the first genetically modified crop in the last decade to be approved for cultivation in Europe.

Functional quality

The paste made from potato flour is characterized by low elasticity and its use in the food industry is rather limited. Potato plants carrying a gene for low-weight glutenin (LMW-GS-MB1) have been produced with the aim of improving the functional qualities of potato flour. The source of the gene is wheat (*Triticum aestivum*). The LMW-GS-MB1 units accumulated in transgenic tubers function as a polymer and lead to a threefold increase in potato flour viscosity (Benmoussa et al., 2004).

Carotenoid level

In a drive for the nutritional improvement of foods, scientists in Scotland have developed a transgenic potato plant with enhanced carotenoid content in the tubers. These isoprenoid pigments, found in photosynthetic tissues and in many flowers and fruits, are generally lacking in the seeds and storage organs that form common staples. They are, however, dietary essentials. Carotenoid enhancement could have an impact on health (Laurence et al., 2005). The enzyme phytoene synthase provides the gateway to biosynthesis through the conversion of the C20 isoprenoid geranylgeranyl pyrophosphate to the C40 proto-carotenoid phytoene. In growing tubers of the transgenic plants a phytoene synthase gene from the bacterium *Erwinia uredovora* raised the total carotenoids up to sixfold.

Production of vaccines and human proteins

The majority of recombinant proteins are produced by microorganisms, and transgenic plants represent an alternative system for their production. The most suitable plant for the production of human vaccines (edible vaccines) seems to be the banana plant. However, tomato, maize and potato plants are more frequently used as model systems in typical experiments dealing with the expression of foreign proteins in plants (Stirn and Lorz, 2003).

Plant-based edible vaccines are plant materials administered via the oral route, prepared by using transgenic plants that express antigen proteins capable of inducing protective immunity against various human and animal diseases to induce specific immune responses. Usually, vaccination is a process by which immune responses against pathogens are induced without real infection and can protect the hosts against pathogenic infection. Immunology textbooks currently report orally administered antigens as inducing immune tolerance rather than immune stimulation. Nevertheless, current plant-based edible vaccine technology, if sufficiently developed, may offer several advantages. For example, it is easy to apply, store and transport. It could also induce both mucosal and systemic immune responses, which cannot be achieved using an injected vaccine. Plant-based vaccines are also anticipated to prove useful in the animal industry, since the cost of injection is significant. Although no commercial plant-based edible vaccines are currently available, several candidate vaccines are undergoing clinical trials. Consequently, many scientists are anticipating that a commercial plant-based edible vaccine will be available in the near future (Kim and Yang, 2010). To illustrate this some of the vaccines and human proteins produced from transgenic potato plants are as follows:

Viral diseases of humans:

a) Vaccine against hepatitis B (Kong et al., 2001).
b) Vaccine against Norwalk virus (Mason et al., 1996; Tacket et al., 2000).

c) Vaccine against papillomaviruses (Biemelt et al., 2003).
d) Vaccine against hantaviruses (Murray et al., 2002; Kehm et al., 2001).

Viral diseases of animals:

a) Vaccine against lethal rabbit hemorrhagic disease (RHDV) (Castanon et al., 1999; Martin-Alonso et al., 2003).
b) Vaccine against infectious bronchitis virus (IBV) (Zhou et al., 2003; 2004).
c) Vaccine against rotaviruses (Matsumura et al., 2002; Yu and Langridge, 2003).
d) Vaccine against infectious gastroenteritis (TGEV) (Gomez et al., 2000).
e) Vaccine against foot and mouth disease (Carrillo et al., 2001).

Vaccines against bacterial diseases of humans and animals:

a) Vaccine against enterotoxigenic strains of *E. coli* (ETEC) (Mason et al., 1998; Lauterslager et al., 2001).
b) Vaccine against the toxin of *Vibrio cholerae* (Arakawa et al., 1997).

Production of human proteins:

a) Production of human lactoferrin (Chong and Langridge, 2000).
b) Production of human interferons (HuIFN-a-2b and HuIFN-a-8) (Ohya et al., 2001).
c) Production of human tumour-necrotizing factor (HuTNF-a) (Ohya et al., 2002; Streatfield and Howard, 2003).

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Review

PHYTOREMEDIATION: A NOVEL GREEN TECHNOLOGY TO RESTORE SOIL HEALTH

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The widespread occurrence of moderate levels of metal pollution, which is increasing both in India and worldwide, has led to limitations in land use, and soil remediation is needed. Phytoremediation, the use of plants for cleaning metal-polluted soils, is of low cost, environmentally sound and equally protective of human health and the environment, and should be considered a good alternative to current techniques. Brassica, a crop widely grown in North India, can be effectively used for remediating the soil with no change in the agricultural systems followed by the farmers. Phytoremediation is a word formed from the Greek prefix "phyto" meaning plant, and the Latin suffix "remedium" meaning to clean or restore (Cunningham et al., 1997). The term actually refers to a diverse collection of plant-based technologies that use either naturally occurring or genetically engineered plants for cleaning contaminated environments (Flathman and Lanza, 1998).

Some plants which grow on metalliferous soils have developed the ability to accumulate massive amounts of the indigenous metals in their tissues without exhibiting symptoms of toxicity (Baker and Brooks, 1989; Baker et al., 1991; Reeves and Brooks, 1983). Chaney (1983) was the first to suggest using these "hyperaccumulators" for the phytoremediation of metal-polluted sites. However, hyperaccumulators were later believed to have limited potential in this area because of their small size and slow growth, which limit the speed of metal removal (Comis, 1996; Cunningham et al., 1995; Ebbs et al., 1997). By definition, a hyperaccumulator must accumulate at least 100 mg g^{-1} (0.01% dry wt) Cd, As or some other heavy metals, 1000 mg g^{-1} (0.1% dry wt) Co, Cu, Cr or Pb and 10,000 mg g^{-1} (1% dry wt) Mn or Ni (Reeves and Baker, 2000; Watanabe, 1997).

Some plants tolerate and accumulate high concentrations of metal in their tissues, but not at the level required to be called hyperaccumulators. These plants are often called moderate metal-accumulators, or just moderate accumulators (Kumar et al., 1995). The lack of viable plant alternatives for phytoremediation seemed to suppress the amount of phytoremediation research conducted between the mid 1980s and the early half of the 1990s. The search for plants for phytoremediation centred on the Brassicaceae family to which many hyperaccumulators belong (Cunningham et al., 1995).

A survey of the literature suggests that crops such as Indian mustard and maize possess the ability to hyperaccumulate the toxic heavy metals in Indian continental ecosystems (Panwar et al., 2002b; Mishra et al., 2007; Panwar and Marton, 2008; Pal, 2009; Panwar, 2010).

The technology of phytoextraction utilizes hyperaccumulating plants in order to extract heavy metals from the environment. Most hyperaccumulating plants belong to the families Brassicaceae (pl. *Thlaspi*, *Alyssum*), Compositae, Euphorbiaceae, Fabaceae, Liliaceae, Scrophulariaceae, Poaceae and Violaceae, which also occur in the native Hungarian flora. Hardly any of them are agricultural plants, so their economic utilization is problematic (also often because of low biomass and complicated harvesting and propagation). Another problem is the selectivity of accumulation. Various innovations have attempted to solve these problems, e.g. the heavy metal uptake of non-hyperaccumulating plants with large biomass can be enhanced by means of chelating soil treatment, for instance using EDTA (Huang et al., 1997).

Tobacco (*Nicotiana tabacum*) has been proved to easily accumulate heavy metals, particularly cadmium, in the leaves, which is then transferred to the smoke. The accumulation and uptake of heavy metals by flue-cured tobacco depends upon the soil properties and the genetic characters of the plants. Higher values of leaf heavy metal concentrations were observed in the North-Eastern regions of Hungary, where the soils are highly acidic in nature (Gondola and Kádár, 1995). Tobacco may thus be a promising phytoremediating hyperaccumulator plant species in regions with contaminated acidic soils. The metal hyperaccumulator *Thlaspi caerulescens* also has a wide variety of metal uptake and translocation characteristics (Baker and Brooks, 1989).

Phytoremediation consists of a collection of five different plant-based technologies, each having a different mechanism of action for the remediation of metal-polluted soil, sediment or water. These include: rhizofiltration, which involves the use of plants to clean various aquatic environments; phytostabilization, where plants are used to stabilize rather than clean contaminated soil; phytovolatilization, which involves the use of plants to extract certain metals from soil and then release them into the atmosphere through volatilization; and phytoextraction, where plants absorb metals from soil and translocate them to the harvestable shoots where they accumulate.

Phytoremediation is defined as the use of green plants to partially or substantially remediate selected contaminants in contaminated soil, sludge, sediment, groundwater, surface water and waste water. It utilizes a variety of plant biological processes and the physical characteristics of plants to aid *in situ* remediation. Phytoremediation has also been called green remediation, botano-remediation, agroremediation and vegetative remediation. The conventional methods of remediation may cost from $10 to $1000 per cubic metre. Phytoextraction costs are estimated to be as low as $0.05 per cubic metre (Cunningham et al., 1997). Phytoremediation is a continuum of processes, with the different processes occurring to differing degrees in different conditions, media, contaminants and plants.

Types of phytoremediation

Rhizofiltration

Metal pollutants in industrial waste water and in the groundwater can be effectively removed by rhizofiltration. The process involves raising plants hydroponically and transplanting them into metal-polluted waters, where the plants absorb and concentrate the metals in their roots and shoots (Dushenkov et al., 1995; Flathman and Lanza, 1998; Salt et al., 1995; Zhu et al., 1999). Root exudates and changes in rhizosphere pH may also cause metals to precipitate onto root surfaces. As they become saturated with the metal contaminants, roots or whole plants are harvested for disposal (Flathman and Lanza, 1998; Zhu et al., 1999). Several aquatic species have the ability to remove heavy metals from water, including water hyacinth [*Eichhornia crassipes* (Mart.) Solms] (Kay et al., 1984; Zhu et al., 1999), pennywort (*Hydrocotyle umbellata* L.: Dierberg et al., 1987) and duckweed (*Lemna minor* L.: Mo et al., 1989).

However, these plants have limited potential for rhizofiltration, because they are not efficient at metal removal, a result of their small, slow-growing roots (Dushenkov et al., 1995). Terrestrial plants are thought to be more suitable for rhizofiltration because they produce longer, more substantial, often fibrous root systems with large surface areas for metal sorption. Sunflower (*Helianthus annuus* L.) and Indian mustard (*Brassica juncea* Czern.) are the most promising terrestrial candidates for metal removal in water. The roots of Indian mustard are effective for the removal of Cd, Cr, Cu, Ni, Pb and Zn (Dushenkov et al., 1995), and sunflower removes Pb (Dushenkov et al., 1995), U (Dushenkov et al., 1997a), ^{137}Cs and ^{90}Sr (Dushenkov et al., 1997b) from hydroponic solutions. Indian mustard has proved to be effective in removing a wide concentration range of lead (4–500 mg/l) (Raskin and Ensley, 2000).

Thus, rhizofiltration is defined as the use of plants, both terrestrial and aquatic, to absorb, concentrate and precipitate contaminants from polluted aqueous sources with low contaminant concentration in their roots. Rhizofiltration can partially treat industrial discharge, agricultural runoff or acid

mine drainage. It can be used for lead, cadmium, copper, nickel, zinc and chromium, which are primarily retained within the roots (Chaudhry et al., 1998; US EPA, 2000).

Phytostabilization

Phytostabilization, also known as phytorestoration, is a plant-based remediation technique that stabilizes wastes and prevents exposure pathways via wind and water erosion. It provides hydraulic control, which suppresses the vertical migration of contaminants into the groundwater, and physically and chemically immobilizes contaminants by root sorption and by chemical fixation with various soil amendments (Berti and Cunningham, 2000; Flathman and Lanza, 1998; Salt et al., 1995; Schnoor, 2000). Plants chosen for phytostabilization should be poor translocators of metal contaminants to aboveground plant tissues that could be consumed by humans or animals. The lack of appreciable metals in shoot tissue also eliminates the necessity of treating harvested shoot residue as hazardous waste (Flathman and Lanza, 1998).

The selected plants should be easy to establish and care for, grow quickly, have dense canopies and root systems, and be tolerant of metal contaminants and other site conditions which may limit plant growth. The research of Smith and Bradshaw (1979) led to the development of two cultivars of *Agrostis tenuis* Sibth. and one of *Festuca rubra* L., which are now commercially available for the phytostabilization of Pb-, Zn- and Cu-contaminated soils. Phytostabilization is most effective at sites having fine-textured soils with high organic matter content, but is suitable for treating a wide range of sites where large areas of surface contamination exist (Berti and Cunningham, 2000; Cunningham et al., 1995).

Phytovolatilization

Some metal contaminants such as As, Hg and Se may exist as gaseous species in the environment. Some naturally-occurring or genetically modified plants are capable of absorbing elemental forms of these metals from the soil, biologically converting them to gaseous species within the plant, and releasing them into the atmosphere. This process is called phytovolatilization, the most controversial of all phytoremediation technologies. According to Brooks (1998), the release of volatile Se compounds from higher plants was first reported by Lewis et al. (1966).

Terry et al. (1992) reported that members of the Brassicaceae are capable of releasing up to 40 g Se ha^{-1} day^{-1} as various gaseous compounds. Some aquatic plants, such as cattail (*Typha latifolia* L.), are also good for Se phytoremediation (Pilon-Smits et al., 1999). Unlike the plants that are used for Se volatilization, those which volatilize Hg are genetically modified organisms. *Arabidopsis thaliana* L. and tobacco (*Nicotiana tabacum* L.) have been genetically modified with bacterial organomercurial lyase (merB) and mercuric reductase (merA) genes (Heaton et al., 1998; Rugh et al., 1998). These plants absorb Hg(II) and

methylmercury (MeHg) from the soil, while Hg(0), i.e. elemental mercury, can be volatilized by the cells into the atmosphere (Heaton et al., 1998).

Phytoextraction

Phytoextraction is the most commonly recognized of all phytoremediation technologies. The phytoextraction process involves the use of plants to facilitate the removal of metal contaminants from a soil matrix (Kumar et al., 1995). In practice, metal-accumulating plants are seeded or transplanted into metal-polluted soil and are cultivated using established agricultural practices. The roots of established plants absorb metal elements from the soil and translocate them to the aboveground shoots, where they accumulate. If metal availability in the soil is not adequate for sufficient plant uptake, chelates or acidifying agents may be used to liberate them into the soil solution (Huang et al., 1997; Lasat et al., 1998). After sufficient plant growth and metal accumulation, the aboveground portions of the plant are harvested and removed, resulting in the permanent removal of metals from the site.

Several approaches have been used, but the two basic strategies of phytoextraction which have finally developed are: i) Chelate-assisted phytoextraction or induced phytoextraction, in which artificial chelates are added to increase the mobility and uptake of metal contaminants; ii) Continuous phytoextraction, where the removal of metal depends on the natural ability of the plant to remediate; only the number of plant growth repetitions is controlled (Salt et al., 1995; 1997). As with soil excavation, the disposal of contaminated material is a concern. Some researchers suggest that the incineration of harvested plant tissue dramatically reduces the volume of the material requiring disposal (Kumar et al., 1995). In some cases valuable metals can be extracted from the metal-rich ash and serve as a source of revenue, thereby offsetting the expense of remediation (Comis, 1996; Cunningham and Ow, 1996).

Ebbs et al. (1997) reported that *B. juncea*, while having one-third the concentration of Zn in its tissue, is more effective at Zn removal from the soil than *Thlaspi caerulescens*, a known hyperaccumulator of Zn. This advantage is due primarily to the fact that *B. juncea* produces ten times more biomass than *Thlaspi caerulescens*. Plants being considered for phytoextraction must be tolerant of the targeted metal or metals, and be efficient at translocating them from roots to the harvestable aboveground portions of the plant (Blaylock and Huang, 2000).

Phytodegradation

Phytodegradation is the breakdown of the organics taken up by the plant to simpler molecules that are incorporated into the plant tissues (Chaudhry et al., 1998). Plants contain enzymes that can break down and convert ammunition wastes, chlorinated solvents such as trichloroethylene and other herbicides. The enzymes are usually dehalogenases, oxygenases and reductases (Black, 1995).

The various methods of phytoremediation are summarized in Table 1, and the suitability of various plant species for the removal of certain elements in Table 2.

Table 1
Soil remediation (Schnoor, 2002)

Application	Description	Contaminants	Types of plants
Phytotransformation	Sorption, uptake and transformation of contaminants	Organics, including nitroaromatics and chlorinated aliphatics	Trees and grasses
Rhizosphere biodegradation	Microbial biodegradation in the rhizosphere stimulated by plants	Organics; e.g. PAHs, petroleum hydrocarbons, TNT, pesticides	Grasses, alfalfa, many other species including trees
Phytostabilization	Stabilization of contaminants by binding, holding soils and/or decreased leaching	Metals, organics	Various plants with deep or fibrous root systems
Phytoextraction	Uptake of contaminants from soil into roots or harvestable shoots	Metals, inorganics, radionuclides	Variety of natural and selected hyperaccumulators, e.g. *Thlaspi, Brassica*
Vegetative crops	Use of plants to retard leaching of hazardous compounds from landfills	Organics, inorganics, wastewater, landfill leachate	Trees such as poplar, plants (e.g. alfalfa) and grasses

Table 2
Partial listing of plants and the chemicals they can remediate

Plant	Chemicals
Arabidopsis	Mercury
Bladder campion	Zinc, copper
Brassica family (Indian mustard & broccoli)	Selenium, sulphur, lead, cadmium, chromium, nickel, zinc, copper, cesium, strontium
Buxaceae (boxwood)	Nickel
Compositae family	Cesium, strontium
Euphorbiaceae	Nickel
Tomato plants	Lead, zinc, copper
Trees in the *Populus* genus (poplar, cottonwood)	Pesticides, atrazine, trichloroethylene (TCE), carbon tetrachloride, nitrogen compounds, 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5 triazine (RDX)
Pennycress	Zinc, cadmium
Sunflower	Cesium, strontium, uranium
Genus *Lemna* (Duckweed)	Explosives wastes
Parrot feather	Explosives wastes
Pondweed, arrowroot, coontail	TNT, RDX
Perennial rye grass	Polychlorinated phenyls (PCPs), polyaromatic hydrocarbons (PAHs)

Indian mustard in phytoremediation

Two *Brassica* species, *B. juncea* and *B. carinata*, or Indian mustard, were grown in an artificially Ni-contaminated soil to study their tolerance and Ni-accumulation. The chelating agent EDTA was applied at the rosette stage to enhance metal uptake. Nickel concentrations almost double that in the control were observed in both the species when Ni contamination was combined with EDTA application. The species *B. juncea* appeared to be slightly more tolerant and to be a better accumulator of Ni. In agreement with earlier reports, the translocation of the pollutant metal to the shoot from the root seemed to be restricted in both the *Brassica* species to higher rates of Ni application plus EDTA.

The results of the study indicated that *B. juncea* has the potential to be a hyperaccumulator of Ni (Panwar et al., 2002b). *Brassica* species can grow well in soil freshly contaminated with 20 and 40 mg Cd kg^{-1} and have high potential for removing Cd from the soil in the course of phytoremediation. *B. juncea* performed better as a hyperaccumulator for cadmium, producing higher biomass than *B. carinata*. EDTA proved to be an effective chelating compound for mobilizing the metal from the soil, enhancing the Cd concentration and uptake in Indian mustard (Ahmed et al., 2001).

The Indian mustard plant (*B. juncea*) can extract both heavy metals and radionuclides from soil. It is a high-biomass crop that has been traditionally grown in Southeast Asia as a source of cooking oil. It is given considerable attention by present-day researchers, geneticists and plant breeders in particular, because of its unique polyploid genome. It is an allotetraploid, a plant with a genome composed of the complete diploid genomes of both parents, *B. nigra* and *B. campestris*. In modern breeding programmes, the selection of *B. juncea* is based on a wide variety of characters. Improving the oil and meal quality by eliminating nutritionally undesirable erucic acid or by modifying the fatty acid composition of the oil is an important objective for plant breeders (Banga, 1997).

The ARS Water Management Research Laboratory in Fresno, California, has had success in using Indian mustard to dramatically reduce selenium levels in soil. Making Indian mustard part of a proper crop rotation can help control selenium levels and minimize the selenium load deposited into the agricultural effluent. In addition, some of the harvested mustard can be blended with hay and fed to animals in nearby areas where selenium deficiency is a problem. In order to see if the *Brassica* species used for selenium uptake could be used as viable oil crops, scientists are currently evaluating the effects of higher selenium concentrations on the oil content.

Based on the Indian mustard germplasm collected by ARS, studies conducted by Phytotech, Rutgers University, and the International Institute of Cell Biology have also shown that Indian mustard has the ability to accumulate heavy metals such as lead, chromium, cadmium, nickel and zinc. The approach requires adding a chelating agent to the soil to solubilize the soil lead and allow

it to move from the roots into the shoots. Phytotech has also had some success in using Indian mustard to remove radionuclides such as cesium-137 and strontium-90 at a site near Chernobyl.

Norman Terry, professor of plant biology at the University of California at Berkeley, is exploring the possibility of using Indian mustard to remove naturally-occurring selenium from the soil. Although a necessary nutrient, selenium may leach into water. In high amounts, this metal may poison wildlife and livestock. In laboratory research, Terry has found that Indian mustard not only takes up selenium but converts it into dimethyl selenide, a gas which he describes as relatively non-toxic. They are currently working on genetically altering the plants to increase the volatilization. According to Terry, there are huge amounts of this gas in the atmosphere from volcanoes, soil and plants, and it is continually recycled; therefore, the amount that would be added via phytoremediation would be negligible.

Inorganic and organic chelates in phytoremediation

Ahmed et al. (2001) reported that when added to soils the chelating agent ethylenediamine tetra acetic acid (EDTA) increased the solubility of heavy elements for plant uptake during phytoremediation. The results indicated that EDTA made the cadmium more readily available to the plants and lowered the Cd content of the soil. The magnitude of the increase in tissue (stem, leaf and root) Cd concentration was higher in *B. juncea* than in *B. carinata* and after the application of the chelating agent (EDTA). The *B. juncea* species of Indian mustard has better potential for the phytoremediation of soil heavily contaminated with Cd (40 mg Cd kg^{-1} soil).

The use of synthetic chelating agents such as EDTA, NTA (nitrilotriacetic acid), etc. is known to promote metal extractability, increased availability to plants and transformation to other forms/complexes. However, as synthetic ligands, especially EDTA, are resistant to microbial decomposition, soil-borne natural agents are preferred. Further, in a developing country like India, the use of synthetic chelating agents on a commercial scale to mobilize heavy metals is uneconomical. A change in soil pH (i.e. lowering it towards the acidic range, using organic acids or organic manure to produce organic acids during the decomposition process) is thus another option to promote the mobility of heavy metals (Panwar and Marton, 2008).

A screen-house experiment was conducted to evaluate the effect of chelating agents (EDTA and NTA), farmyard manure (FYM) and herbicides (Topic and isoproturon) on the phytoextraction ability of Indian mustard (*B. juncea* L.) from soil enriched with Cd (60 μg/g soil). The results indicated that EDTA treatment led to lower biomass production as compared to NTA and FYM. Topic enhanced the biomass yield in comparison to the control and isoproturon. Biomass production was lowest in the isoproturon treatment. The

Cd concentration increased significantly in both roots and shoots with the application of complexing agents, i.e. FYM, EDTA or NTA. The highest plant concentration was recorded in NTA-treated soil. Treatment combinations of FYM, EDTA and NTA with Topic spray led to a higher uptake of Cd into the plant system. Amongst these combinations, NTA–Topic was the most efficient (Mishra et al., 2007).

The effect of cadmium (Cd) contamination on the phytoavailability of micronutrients with or without EDTA amendment in a sandy loam soil artificially contaminated with 40 mg Cd/kg soil was studied using two species of *Brassica, B. juncea* (T-59) and *B. carinata* (HC-2), as test crops. EDTA was applied at 1.0 g/kg soil (as the disodium salt) in solution form at the rosette stage and the plants were harvested at the blooming stage (8 weeks after emergence). EDTA addition caused a slight reduction in biomass production, whereas cadmium contamination caused a significant reduction in the yields of all plant parts with or without EDTA. There was a several-fold increase in both the concentration and uptake of Cd in different plant parts of Indian mustard.

Zinc content and uptake in the stem portion increased by around 50%, whereas the total Zn uptake decreased with Cd contamination, irrespective of the EDTA treatment. However, the Zn uptake in the roots decreased, suggesting its greater translocation to aboveground parts. Iron showed a three- to four-fold increase in the stems of both cultivars, indicating its entrapment in this plant part. Iron uptake by the roots decreased with Cd contamination, but the manganese content in all the plant parts increased. In general the addition of EDTA decreased the total Mn uptake in both the species, indicating the possible competition of Cd with Mn for the binding sites of the chelating agent. *B. juncea* promised to be a relatively better accumulator of both toxic and trace metal ions when combined with EDTA application (Panwar and Marton, 2008).

Because of its strong chelating capacity, the application of EDTA to soils may change the amount and distribution of heavy metals among their various chemical forms. Therefore, a greenhouse experiment was conducted using *B. juncea* and *B. carinata* as hyperaccumulator test crops on natural and artificially Cd- and Ni-contaminated soils. Both the natural and metal-amended soils were treated with the disodium salt of EDTA at 0 and 1 g kg^{-1} soil.

After harvesting the crops, soil samples were fractionated into water-soluble plus exchangeable (WE), carbonate (CARB), organic matter (OM), Mn oxide (MnOX), amorphous Fe oxide (AFeOX), crystalline Fe oxide (CFeOX) and residual (RES) fractions. In metal-amended soils, Cd and Ni were found predominantly in the AFeOX fraction in the absence of EDTA application and in the WE fraction in EDTA treated soil. The application of EDTA resulted in the redistribution of Cd among different forms and Cd increased significantly in the WE fraction, with a concomitant significant decrease in the OM fraction. In natural soils, more than 40% of the total Cd was present in the RES fraction, while in contaminated soil this was only 5%.

Nickel increased significantly in the WE fraction, while it decreased considerably in the CARB, OM, MnOX, AFeOX and CFeOX fractions after EDTA addition. This indicated that EDTA is capable of moving Cd and Ni from the less soluble or more stable forms (CARB, OM, MnOX, AFeOX and CFeOX) to the most soluble form (WE). In natural soils, up to 49% of the Ni was found in the RES fraction, whereas only 10% of the total Ni was observed in this fraction in contaminated soil, irrespective of the EDTA treatment. In general, the amount of Cd recovered after the harvest of both *Brassica* species did not differ significantly in any fraction except the WE fraction. The amount of Ni recovered in the AFeOX fraction was significantly higher in *B. juncea* than in *B. carinata* (Panwar et al., 2002a; 2005).

Microbial biomass in phytoremediation

The growth of *B. juncea* was better in soil amended with farmyard manure (FYM) or vermicompost (VC) as compared to unamended Cd-enriched soil. Growth was slightly suppressed in EDTA-treated soil, whereas it was better in soil treated with bioinoculants. The application of FYM and VC increased the dry matter yield of Indian mustard either alone or in combination with bioinoculants. The application of EDTA caused a significant decrease in the biomass of Indian mustard, while that of bioinoculants increased the dry matter yield of both roots and shoots, but not significantly, because the bioinoculants showed greater sensitivity towards cadmium.

The cadmium concentration was observed to be maximum when EDTA was combined with bioinoculants, while the Cd uptake was maximum in the vermicompost + bioinoculant treatments. Compared with the Cd_{100} treatment the Cd concentration in the shoot increased by 120% in Cd_{EDTA}, followed by Cd_{VC} (65%) and Cd_{FYM} (42%) in the absence of microbial inoculates. The corresponding values in the presence of microbial inoculates were 107, 51 and 37%, respectively. A similar trend was observed in the roots, with the order $Cd_{EDTA+M} > Cd_{VC+M} > Cd_{FYM+M} > Cd_{100+M}$. Increases of 5.5% in the root and 4.1% in the shoot Cd content were observed with microbial inoculates in the Cd_{EDTA+M} treatment compared with the Cd_{EDTA} treatment. FYM, Vermicompost and EDTA also increased the Cd uptake significantly with and without microbial inoculates in the shoots and roots. The results indicated that Vermicompost in combination with bioinoculants is a better solution for the phytoremediation of Cd by Indian mustard from Cd-contaminated soil, giving the highest uptake value in the shoot in the Cd_{VC+M} treatment (2265.7 μg/pot), followed by Cd_{EDTA+M} (2251.2 μg/pot), Cd_{FYM+M} (1485.7 μg/pot) and Cd_{100+M} (993.1 μg/pot) (Panwar, 2010).

Simon et al. (2008) reported that crop species removed considerable amounts of cadmium from solution containing Cd after 48 hours of exposure. Most of the Cd was accumulated in the roots of the plants; the rate of Cd translocation to the shoots was low. The efficiency of Cd removal from

contaminated solution was proportional to the root dry matter content (biomass production) of the plants. Pre-treatment of Indian mustard with the *Pseudomonas fluorescens* bacterium before cadmium and nickel application slightly enhanced the Cd or Ni accumulation in the roots. This enhancement was not observed when pseudomonads were applied simultaneously with the metals.

More cadmium was detected in the roots of Indian mustard when the plants were pre-treated with Cd-tolerant *Pseudomonas cepacia* than with the Cd-sensitive variant. The cell number of pseudomonads on the surface of Cd-treated Indian mustard roots was significantly lower when the plants were pre-treated with Cd-sensitive *P. cepacia*. In spite of the occurrence of new root hairs, the pre-treatment of the roots with the plant hormone ethylene proved to be ineffective in enhancing the rhizofiltration capacity of Indian mustard, since root elongation stopped and the dry matter production of the roots was reduced. Only the *Bacillus* and *Pseudomonas* species (plant growth-promoting rhizobacteria, PGPR) present in Se-contaminated soils can be expected to stimulate Se phytoextraction and/or phytovolatization in the rhizosphere of higher plants (Simon et al., 2007)

The abundance and infection frequency of colonizing arbuscular (AM) mycorrhiza fungi on different cultivated plants, and the mycorrhizal effect of inorganic and organic nutrient additives were investigated on recultivated mine spoils from different geological layers. Several publications report on the role of ecto- and endomycorrhizal fungi in the recolonization of heavy metal-contaminated soils by pioneer plants. Mycorrhizal fungi offer a better nutrient supply and higher metal tolerance to the macrophytic symbiotic partner. The arbuscular type of mycorrhiza (AM) is more abundant and more ancient, and offers better soil exploitation, and higher nutrient and water uptake, thus resulting in higher biomass production.

AM fungi are among the most abundant soil fungi. The effect of AM fungi on the heavy metal uptake of the host plant depends on the physical and chemical properties of the contaminated soil, on the severity and duration of the contamination load and on the plant and fungal species, and thus on the efficiency of the symbiosis. The published data demonstrate that by the selected coupling of compatible symbiotic partners, plant metal uptake can be altered parallel to higher plant vitality.

In the case of beneficial microbes, such as the nitrogen-fixing *Rhizobium* bacteria, or the phoshorus-mobilising arbuscular mycorrhizal fungi (AMF), there is a discrepancy in the literature regarding their metal tolerance and their functions in plant performance and plant fitness (Biró et al., 1998; Takács et al., 2006). Reliable long-term field experiments, which are infrequent in Europe, can therefore serve as valuable tools to study these effects and the behaviour of heavy metals under natural conditions (Kádár and Németh, 2005).

Other studies mainly show the direct effect of the metals, but due to the multifactorial nature of environmental conditions, only long-term experiments

can show both the abundance and the functioning of plant–microbe interactions. The effect of heavy metals is known to be different if other amendments, such as organic or inorganic additives or soil conditioners, are applied to metal-polluted soils (Posta and Füleky, 1997; Simon and Biró, 2005). The combination of these procedures with beneficial metal-tolerant microbes and/or microsymbionts may have further benefits, such as stabilising the metals in the rhizosphere and improving the nutrient supply or the growth and fitness of the higher plants (Simon and Biró, 2005; Vivas et al., 2006). Under field conditions metal- or salt-tolerant abilities were found to exhibit seasonal and annual patterns (Biró et al., 1999; Naár and Biró, 2006; Füzy et al., 2006).

Depending on the severity of the stress, beneficial microbes could confer stress tolerance on their macrosymbiont hosts (Vivas et al., 2006), particularly if they are well-adapted to the given environment. Other findings with non-adapted fungi have also shown the potential applicability of symbiosis in other methods of phytoremediation, such as phytoextraction (Takács and Vörös, 2003).

Biotechnology in phytoremediation

Scientists have engineered tree tobacco (*N. glauca*) to contain a wheat gene that makes yeast resistant to cadmium. The over-expression of this gene made the tobacco more resistant to Pb (Gisbert et al., 2003).

Using *Agrobacterium tumefaciens*, the plant *Arabidopsis thaliana* was engineered to contain XplA, a bacterial gene that can degrade RDX. Scientists found that the engineered plants degraded RDX better than the control plants (Rylott et al., 2006).

Scientists increased the tolerance of tobacco to heavy metals by engineering it to produce a polyhistidine peptide that has the ability to bind to various heavy metal ions, creating an "artificial sink" (Shingu et al., 2006).

Even though hyperaccumulators are very efficient for the absorption of heavy metals, most are unsuitable for phytoextraction because they lack other characteristics important for phytoextraction, such as fast growth rate and large biomass (Cherian and Oliveira, 2005).

In 2004, scientists published the results of a study on *T. caerulescens* (alpine pennycress), a very effective hyperaccumulator, and identified the genes responsible for tolerance of heavy metals and hyperaccumulative ability (Ashot and Kochian, 2004).

The level of metal uptake by flue-cured tobacco (*N. tabacum*) was found to depend on the genotype (Kadar, 1992).

The advantages and disadvantages of phytoremediation are summarized in Table 3.

Table 3
Advantages and disadvantages of phytoremediation

No	Advantages	Disadvantages/Limitations
1	Amendable to a variety of organic and inorganic compounds	Restricted to sites with shallow and low contamination within the rooting zone of remediative plants.
2	*In situ*/*ex situ* application possible with effluent/soil substrate, respectively.	May take up to several years to remediate a contaminated site.
3	*In situ* applications decrease the amount of soil disturbance compared to conventional methods.	Restricted to sites with low contaminant concentrations.
4	Reduces the amount of waste to be landfilled (up to 95%); can be further utilized as bio-ore of heavy metals.	Harvested plant biomass from phytoextraction may be classified as a hazardous waste, requiring suitable disposal.
5	*In situ* applications decrease the spread of the contaminant via air and water.	Climatic conditions are a limiting factor
6	Does not require expensive equipment or highly specialized personnel.	Introduction of non-native species may affect biodiversity
7	In large-scale applications the potential energy stored can be utilized to generate thermal energy.	Consumption/utilization of contaminated plant biomass is a cause of concern.

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Obituary

PROF. JÓZSEF SUTKA
(1936–2010)

With the death of József Sutka, the Hungarian and international scientific community has lost a well-known and much honoured member.

József Sutka began his education in a small village school, but thanks to his outstanding diligence and ability he was granted a place at the best grammar school in the southern Hungarian city of Szeged, where he continued to make good progress. He started his university studies at the University of Agricultural Sciences in Gödöllő, but then obtained a scholarship to the Faculty of Biology and Soil Science of Leningrad University, where he received a first class degree in genetics in 1961. He also acquired a fluent knowledge of Russian, which he made good use of during his long years in the Martonvásár institute.

After obtaining his degree he worked in the Plant Breeding Department of Gödöllő University from 1961 to 1971, specialising in mutation genetics and breeding. After a few months in the newly established Biology Centre in Szeged, he started work in the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár on May 1st 1972, becoming one of the founding members of the research staff engaged to work in the newly constructed phytotron.

He started work on wheat cytogenetics, a field then gaining momentum worldwide, and specialised in the development of monosomic series. In the course of this time-consuming, labour-intensive work he successfully developed complete monosomic series for two varieties. Later he developed numerous other basic genetic materials (substitutions, recombinant lines), which still form a valuable part of the Martonvásár Cereal Gene Bank. These lines are unique throughout the world and represent an irreplaceable treasure trove for future research on molecular genetics.

The results he achieved in genetic research on wheat frost tolerance laid the foundations of his international reputation. He was the first to determine that the most important gene for frost tolerance, Fr1, was located on chromosome

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5A. This result, published in TAG in 1981 and since cited in almost 100 publications, was later confirmed by molecular genetic analysis. The basic genetic materials he developed provided an excellent foundation for the molecular mapping of the genes responsible for frost tolerance.

His scientific work was recognised in the granting of a university doctorate in 1964, a PhD in biology in 1970 and a DSc in 1989.

For József Sutka, research was more a hobby than a job. It was almost impossible to go into the phytotron in the evenings or at the weekend without finding him there. His exceptional attention to detail led to his visiting his frost tolerance experiments daily, frequently watering and caring for the plants himself. He was also extremely precise in his evaluation of the results. His whole attitude to his work set an excellent example to the next generation of scientists.

Thanks to his great knowledge and reputation for hard work, he was charged with a great many tasks over the years, all of which he completed conscientiously. For many years he was chairman of the Genetics Division of the Hungarian Society for Agricultural Sciences and of the Agriculture jury of the National Scientific Research Fund. Special mention should be made of his activities as the editor of the English language journal, Acta Agronomica Hungarica, from 1996 until 2004. With his characteristic enthusiasm and precision, he ensured that the journal was published regularly, at a high standard.

Right from the beginning, he took an active part in the teaching of cytogenetics, lecturing to both undergraduates and postgraduates, not only in Hungarian but also in English and Russian. He was co-author of eight sets of lecture notes on this subject. In recognition of his work in education, he was awarded the title of Honorary Professor in 1985 and was the recipient of the Pro Universitate medal in 1995.

His first book, entitled Cytogenetics, was published in 1980, and he was just able to finish the second, entitled Plant Cytogenetics, before his illness struck in 2004. These books were warmly received not only in Hungary but also abroad.

His love of teaching also made itself felt in his relationship with young scientists. He not only expected much of them, but also gave them great encouragement and showed his appreciation of their efforts. With his active support, several different projects were initiated in the Genetics Department, which have now developed into separate departments headed by Gábor Galiba (originally Wheat Genetics, now Plant Molecular Biology) and Márta Molnár-Láng (Department of Plant Genetic Resources), and a team headed by Géza Kovács (Cereal Gene Bank and Organic Breeding).

His prestige as an acknowledged expert in his field was indicated by the fact that he was elected to the governing bodies of numerous scientific organisations, including the Genetics Committee of the Hungarian Academy of Sciences and the Association of Hungarian Geneticists, and was also a member of the editorial committees of several scientific journals.

In recognition of his services to breeding-oriented genetic research, he was awarded the Rudolf Fleischmann Prize in 2009.

He was, if anything, more widely appreciated internationally than within Hungary. He was a pillar of EWAC, the international organisation set up in the 1960s to coordinate wheat aneuploid research, which meant that he was in direct personal contact with all the scientists working on wheat cytogenetics in Europe. The 6 months he spent as a visiting scientist in Cambridge in 1976 led to long-term research cooperation and many joint publications. Later he also developed active cooperation and cordial relationships with a number of leading wheat geneticists from the US.

His illness robbed us of a valued colleague, but the genetic materials he developed, together with his books and other publications, will ensure that he is not forgotten by future generations.

The lasting achievements that marked the three decades he spent in the Agricultural Research Institute of the Hungarian Academy of Sciences will be preserved by his successors and utilised for the public good.

M. MOLNÁR-LÁNG

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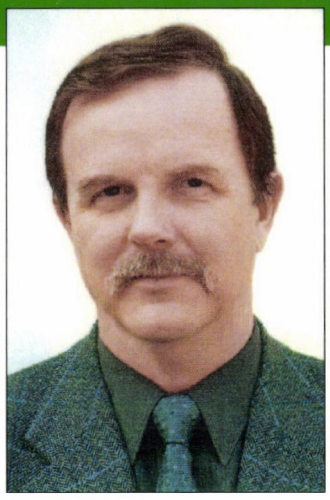

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